EXPERIMENTALLY INDUCED *Vibrio fetus* VAR. *venerealis* INFECTION IN THE GUINEA PIG (*Cavia porcellus* L.)

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A. M. D. G.

To the memory of my parents

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by
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A study of Vibrio fetus var. venerealis infection of the female guinea pig was undertaken to determine the value of this animal as an experimental model and to assess the influence of estrogen and progesterone on the initial establishment and course of the infection.

Estrone in combination with progesterone, when incorporated into the culture medium, had a growth stimulating effect on V. fetus var. venerealis in vitro.

Guinea pigs with normal reproductive cycles failed to develop a prolonged vaginal or systemic infection when inoculated by the intravaginal route with viable V. fetus var. venerealis. The use of exogenous estrogen or progesterone did not influence the course of vaginal infection. The organism was not isolated from the uterus and was rapidly cleared from the vagina. A group of estrous guinea pigs inoculated intravaginally with V. fetus var. venerealis and then mated showed a decrease in litter size and an increase in neonatal deaths when compared to a control group.

When inoculated into the uterus of ovariectomized guinea pigs the organism spread throughout the reproductive tract and was isolated from the blood, liver, spleen, gallbladder and peritoneal cavity. The use of exogenous estrogen or progesterone did not influence the course of the infection, which was transitory. The organism did not reproduce in the female genital tract.

Intrauterine inoculation of the gravid female guinea pig with V. fetus var. venerealis was followed by abortion within 30 hours. A generalized infection of the aborted fetuses was noted together with involvement of the reproductive tract, fetal-maternal membranes, blood, liver, spleen and peritoneal cavity of the dam. Pregnant animals which did not abort revealed transitory vaginal infection and fetal resorption.

No lesions were found on histopathological examination of the uterus of normal intact guinea pigs and ovariectomized guinea pigs. Histopathological examination of the uterus and placenta from the animals which aborted showed necrosis, hemorrhage, edema and inflammatory reaction.

The value of the guinea pig as a model for the study of V. fetus var. venerealis infection was not established under the experimental conditions used. The non-gravid guinea pig was refractory to the infection when inoculated by the vaginal route. The use of intrauterine inoculation in the non-gravid ovariectomized guinea pig resulted in an infection which was well tolerated by the animal and failed to produce pathogenisis. Exogenous hormones produced little effect on the course of the infection. A marked predilection for the gravid uterus was clearly demonstrated and the possible value of the pregnant female guinea

pig as a diagnostic aid was discussed.

The host specificity of V. fetus var. venerealis for the cow was considered to be the factor largely responsible for the failure to establish a lasting and progressive infection in the non-gravid female guinea pig.

INTRODUCTION

Since the first isolation and description of Vibrio fetus by MacFadyean and Stockman (1913), genital vibriosis has become recognized as one of the largest single causes of embryonal loss in cattle (Faulkner, 1968). It is a widespread venereal disease which results in considerable economic loss to the cattle industry both in the United States and abroad.

Both the carrier bull and the infected cow are asymptomatic and remain apparently healthy. Vibriosis may be recognized in a herd by careful appraisal of the breeding records. Indications of the disease are a reduction in calf crop from the previous year and the repeated return of cows to service. The suspected diagnosis is confirmed by means of bacteriological procedures or serological methods (Lovell, 1964).

The specificity of present serological methods for the diagnosis of vibriosis, in either the bull or the cow, is not wholly reliable (Plastridge and Easterbrooks, 1953; Lawson and MacKinnon, 1953). The use of vaginal mucus antibody titers to establish a diagnosis of genital vibriosis in the cow, although more dependable than currently available serological techniques (MacKinnon, 1954), is complicated by the occurrence of false positive results (Boyd, 1955).

In spite of the improvements made in methods of isolation, the diagnosis of V. fetus in the bull remains a key problem and often requires test-mating to virgin heifers with subsequent isolation of the organism from the female reproductive tract (Adler, 1953). In vivo research of

bovine vibriosis is still mainly dependent upon the cow. This factor alone often presents problems of availability, cost and handling which could be avoided if a reliable small laboratory animal were available.

Although Smith (1918) was the first to study the effects of V. fetus infection in small laboratory animals, especially guinea pigs, very little data have been reported since that time to indicate conclusively the value of these animals either as diagnostic aids or in the area of research. At present there is apparently no suitable laboratory animal available for these purposes. The need to evaluate species of laboratory animals for use in vibriosis research was recognized by McEntee (1958).

This study was undertaken to attempt to gain a greater understanding of V. fetus var. veneralis infection in both gravid and non-gravid guinea pigs. Emphasis was also placed upon the influence of the hormonal condition of the female reproductive tract and the route of inoculation on attempts to establish the infection in the reproductive tract.

REVIEW OF LITERATURE

Koch (1891) recognized the causal relationship between a specific microorganism and a given disease and established three general criteria to prove such a relationship. These criteria became popularly known as "Koch's postulates." Certain limitations to the application of the "postulates" were recognized by Wilson and Miles (1964) but in essence they remain as Koch originally proposed them.

The dynamics of a host-parasite relationship were reported by Garber (1960). This author noted that the host must provide all the substrates necessary to satisfy the metabolic and reproductive processes of the microorganism. Lichstein (1959) described cell adaptability, a suitable physical environment and, most importantly, the chemical composition of the medium as important factors in the in vitro initiation of cell growth.

Microbial pathogenicity is the ability of an organism to produce disease in a susceptible host. The importance of in vivo studies of microbial pathogenicity was emphasized by Smith (1960) who noted that the peculiar nutritional and other growth requirements of various pathogens, which led to their tissue specificity in disease processes, were not always reproducible in vitro. He cited Brucella abortus infection of the bovine cotyledon and Corynebacterium diphtheriae infection of the throat and tonsils of man as typical examples.

The role of microorganisms as a cause of reproductive diseases of cattle was first discovered by Bang (1897), with the isolation and

description of Brucella abortus. V. fetus was established as a cause of infectious abortion in cattle by Smith (1919). The organism was isolated from the fetal membranes of infected cows. The possibility of V. fetus as a cause for low conception rates in cattle was first suggested by Plastridge and Williams (1943). Other infectious diseases which influence reproduction include those of viral, fungal and protozoal origin. These are reviewed by Osebold (1969).

The bacterial flora of the reproductive tract may be classified either as "normal" or potentially pathogenic. Bacterial populations of the human vagina were found to be influenced by the hormonal condition of the reproductive system (Huffman, 1959; Jones, Carter, Thomas, Peete and Cherney, 1959). A constant bacteriological finding in the normal human vagina is Doderlein's bacillus (Lactobacillus acidophilus) which, in part, controls the micro-environment of other microorganisms present in the vagina (Hunter, Long and Schumacher, 1959). The presence of potentially pathogenic organisms in the cervical flora of the human pregnant female was noted by White and Koontz (1968). These organisms were considered as a hazard to pregnancy only if certain predisposing factors were present in the host. These factors were an inadequate nutritional status, anemia and difficult parturition. Complications in human pregnancy due to V. fetus infection were described by Vinzent, Delarue and Hebert (1950).

The possibility that infertility in cattle was the result of bacterial flora other than V. fetus was suggested by Hatch, Feenstra and Jennings (1949). Lindley and Hatfield (1952) isolated 26 separate species of bacteria from the bovine uterus in a study of infertile cows. The majority of these organisms were of the genus Neisseria. Such infections were considered non-specific. The concept of a non-specific infection with

the production of endometritis as the major cause for infertility was found untenable by Gibbons, Attleberger, Kiesel and Dacres (1959). These authors described some 90 isolants of bacteria from bovine cervical mucus which were classified into 15 genera. In contrast with Hatch et al. (1949) and Lindley et al. (1952) a higher conception rate was observed among those animals which harbored potential pathogens than those found to be bacteriologically negative or to carry non-pathogens.

The non-pregnant mammalian reproductive system undergoes cyclic change in which the physical and chemical nature of the tissues and lumen fluids is altered. These changes are mediated by steroid hormones. Estrogens were found to give rise to increased vasodilation and increased vascularity of the female genitalia (Hansel and Asdell, 1951). Histological changes in the vaginal epithelium of laboratory animals were described by Meyer and Allen (1933). The production of mucus by the vaginal epithelium was due to the simultaneous action of estrogen and progesterone. The production of cornified epithelial cells in the vagina was observed by Meyer et al. (1933) as the result of an increase in the estrogen level of the reproductive system. The use of vaginal cytology to determine the hormonal condition of the female reproductive system of mammals with short reproductive cycles was recommended by Nalbandov (1964). Asdell (1946) reported the occurrence of vaginal epithelial cornification in the guinea pig at estrus. An increase in leucocytic infiltration into the uterine cavity following ovulation was also noted.

Asdell (1955) described an increase in the growth of the capillary bed with subsequent thickening of the endometrium as a result of estrogen stimulation of the uterus. Deposition of glycogen in the uterine epithelium and musculature was another change observed by Boettiger

(1946). Progesterone was also found to influence the production of a characteristic mucus-saturated stratified epithelium (Emmens, 1969). The effect of progesterone on the non-pregnant uterus was to further increase the endometrial thickening, already started by estrogen, and to cause enlargement and branching of the uterine glands (Kraus, 1926).

The physiological effects of estrogen and progesterone cannot be considered separately in the intact female due to a considerable overlapping in the secretion and action of the two hormones. Thus, the uterus is never acted upon by either hormone alone (Nalbandov, 1964). Nevertheless, an increase in steroid hormone levels brought about by the use of exogenous sources of these hormones has allowed the alteration of reproductive cycle lengths and physical and chemical parameters. Smallwood and Sorensen (1969) administered progestogens to feedlot heifers. At slaughter, animals which were slaughtered 48 hours after the final feeding showed an increase in the vascularity of the uterus. Uterine gland epithelial height increased for a 96-hour period after the final feeding but decreased rapidly thereafter. The presence of a very thick, clotted cervical mucus in some of the heifers was considered abnormal. This condition was not found in the untreated control animals. The continual administration of progestins to heifers, when fed at a level of 0.6 mgm per day, was shown effective in the prevention of estrus (Young, Cundiff and Bradley, 1969).

Carroll(1942) noted an increase in both anaerobic and aerobic glycolysis in uterine tissue as a result of exogenous estrogen stimulation. Studies by Leonard and Knobil (1950) described an increase in the beta-glucuronidase activity of the uterus and vagina of the rat when treated with exogenous estrogen. Tissues of rats treated with estradiol were found to

incorporate labeled glycine into protein more readily *in vivo* (Mueller, 1953). A similar stimulation of protein synthesis *in vitro* was reported by Brooks, Leitheiser, De Loecker and De Wever (1969). Significant increases in the incorporation of labeled glycine and leucine into the proteins of uterine microsomal supernatants were attributed to estrone sulfate. This was the only estrogenic compound found to elicit this particular response.

Histochemical changes within the cells of the mammalian reproductive tract are also influenced by hormonally mediated cyclic activity. Akins, Morrisette and Cardeilhac (1969) reported a progressive rise in the luteal tissue acid phosphatase activity in the pig as the corpus luteum matured. A rapid rise in acid phosphatase activity occurred on day 14 of the cycle and was indicative of impending corpus luteum regression. Endometrial acid phosphatase activity was low throughout the cycle. Alkaline phosphatase activity was decreased during the follicular phase and elevated during the luteal phase of the cycle. Similar findings were reported earlier by Goode, Warnick and Wallace (1965).

The lumen fluids of the mammalian reproductive tract comprise those of the fallopian tubes, uterus, cervix and vagina. These fluids are subject to chemical and physical change during the reproductive cycle. Olds and VanDemark (1957) conducted a series of slaughter experiments on cows in which they determined locational differences in the percentages of ash, total nitrogen, reducing sugars and ether extract. Concentrations of reducing sugars were highest in the oviduct and uterus. Cyclic variations in the sodium, potassium and calcium concentrations of the lumen fluids were also noted. Uterine fluid sodium levels of 277 mgm per 100 ml were present in the luteal phase of the cycle compared to 192 mgm per 100 ml in the follicular and proestrus phases. Potassium levels remained virtually

unchanged in the uterus but were higher in the vagina during the luteal phase. Uterine fluid was found to contain large numbers of extruded epithelial cell nuclei in the luteal phase.

Guay (1966) described a decrease in the calcium level of the cervical secretion of the cow on the day of estrus followed by a further steady decline during the post estrus period. The possible effect of even slight changes in the composition of the lumen fluids on the fertility of cows was reported by Guay and Lamothe (1969). A significant decrease in the cervical fluid sodium concentration of infertile cows was observed when compared with the sodium concentration of the cervical fluids of fertile cows. The reproductive cycles of both test and control cows were of normal duration.

The physical and chemical properties of the lumen fluids of the female reproductive system affect the metabolism of the spermatozoon and the ovum both before and after fertilization, and provide for the survival of the blastocyst before nidation (Nalbandov, 1964). Uterine fluid was found to stimulate spermatozoal respiration and to maintain sperm motility (Murdoch and White, 1968). Olds and VanDemark (1957a) recorded differences in the survival times of motile spermatozoa in lumen fluids collected from different parts of the female bovine reproductive system. Motility persisted for 19 hours in follicular fluids but lasted only seven hours in uterine fluids. Blastocyst survival is progesterone dependent. However, Holland, Calhoun, Harris and Walton (1968) showed indirectly that hyperthyroidism enabled blastocyst survival in the rat. This condition stabilized the alkaline phosphatase levels in the uterus of the progesterone deficient rat.

In a study of the effects of increased body temperature on sheep

Ulberg and Burfening (1967) demonstrated damage to both ovum and spermatozoon due to the physical stress of increased air temperatures. If the physical stress was placed on the animals after fertilization the embryo died *in utero* some time later in its development.

The preimplantation blastocyst depends for survival upon the maintenance of a delicate balance of uterine fluid components. Kar, Engineer, Goel, Kamboj, Dasgupta and Chowdhury (1968) determined the principal effect of the intrauterine contraceptive device (IUD) to be a change in the biochemical composition of the uterine fluid. In studies of IUD fitted parous women a significant increase in the total protein and non-protein nitrogen levels of the uterine fluid was described. Such changes were brought about by the lysis of cast off epithelial cells and infiltrated polymorphonuclear leukocytes. These changes produced an unfavorable environment for the development and survival of the blastocyst. A similar finding was reported by Kar, Goswami, Kamboj and Chowdhury (1964) in ovariectomized IUD fitted rats treated with estrogen. Interference with the response of target organs to estrogen was also noted. IUD mediated interference with gonadotropin secretion in heifers was studied by Bhalla, Menon, Woody and Casida (1969). The IUD fitted animals were found to have significantly heavier pituitary glands and lower luteinizing hormone levels than the control animals.

The presence of potential substrates for bacteria in the lumen fluids of the reproductive tract has already been noted (Olds and VanDemark, 1957; Kar *et al.*, 1968). A basis for the tissue specificity of some bacterial species was studied by Keppie, Williams, Witt and Smith (1965). A growth factor, erythritol, which was found to occur in the placental tissues and fetal fluids of the cow, enhanced the virulence of *B. abortus*

for the guinea pig in vivo. The authors further described growth stimulation of B. abortus when erythritol was used as a substrate in vitro. An earlier study by Pearce, Williams, Harris-Smith, Fitzgeorge and Smith (1962) clearly demonstrated that erythritol enabled B. abortus to survive and multiply in bovine phagocytes.

In vitro studies of V. fetus by Osborne and Bourdeau (1955) indicated an increase in the growth rate of the organism when it was grown in a basal thioglycollate broth with separate additions of progesterone, corpus luteum extracts and testosterone. It was suggested that a possible relationship might exist between steroid hormone levels and V. fetus virulence. Growth stimulation of V. fetus in the presence of 0.005% 17, beta-estradiol was observed by Zemjanis and Hoyt (1960). From manometric studies of possible energy sources used by V. fetus, Alexander (1957) determined that lactate effectively stimulated the growth of this organism. The presence of lactate in bovine follicular fluid was shown by Lutwak-Mann (1954). The presence of lactate in the uterine fluid of the human female was demonstrated by Kar et al. (1968).

Nalbandov (1964) noted distinct differences in the response of uterine tissues to bacterial infection. The uterus was found to be more resistant to infection during the follicular phase of the cycle than during the luteal phase. The presence of antimicrobial substances in extracts of the human endometrium was discussed by Kozinn, Pomerance, Caroline and Taschdjian (1968). Extracts from females were pooled without regard for cyclic variation in chemical composition. When tested in vitro they inhibited the growth of Neisseria gonorrhoeae, N. meningitidis and N. haemolysans. The antimicrobial activity was associated with a fraction of the protein content of the extract. The inhibitory action was not

effective against Salmonella sp., Escherichia sp., Proteus sp., Pseudomonas sp. or Staphylococcus sp.

The apparent hormonal regulation of the uterine defense mechanism was observed to be organ specific by Hawk, Simon, Cohen, McNutt and Casida (1955). It did not extend to other organ defense mechanisms in the body. No significant differences were observed in the numbers of E. coli recovered from the peritoneal cavities of either rabbits in estrus or pseudopregnant rabbits 28 hours after intraperitoneal inoculation. However, more organisms were recovered from the pseudopregnant uterus (progesterone influence) than from the estrous uterus (estrogen influence). The organisms gained access to the uterus of the animal through the fallopian tubes. Inoculations made into the pleural cavities of rabbits (estrous and pseudopregnant) revealed a similar clearance rate existed between the two groups and that the clearance was in no way related to the hormonal condition of the reproductive system.

McDonald, Black, McNutt and Casida (1952) studied the effects of bacteria-free semen and bacteriologically contaminated semen upon the rabbit uterus. Neither the estrous nor the pseudopregnant uterus reacted to the bacteria-free semen. When bacteriologically contaminated semen was used as the inoculum the uterus of the pseudopregnant rabbit developed marked pyometra. In contrast, the uterus of the rabbit at estrus showed only a mild inflammatory reaction.

The development of pyometra in heifers and its absence in "repeat breeder" cows as a response to uterine bacterial infection was described by Black, Ulberg, Kidder, Simon, McNutt and Casida (1953). Heifers inseminated during the postestrual period with an E. coli contaminated semen developed pyometra. The "repeat breeder" cows failed to develop

pyometra after similar insemination. The authors suggested that the "repeat breeder" cows lacked the progesterone levels necessary to inhibit the natural uterine defense mechanism and that this inadequate progesterone level was also responsible for the "repeat breeder" condition.

The effect of direct inoculation of a potentially pathogenic organism into the uterine horn was described by Black, Simon, Kidder and Wiltbank (1954). Both the ligated and unligated uterine horns of estrous rabbits inhibited the growth of E. coli whereas the uterine horns of pseudopregnant rabbits not only supported the growth of E. coli but allowed a significant increase in cell numbers.

Rowson, Lamming and Fry (1953) reported the control of bovine uterine infections by means of exogenous estrogen therapy. The administration of exogenous progestrone was found to promote conditions within the uterus to favor the growth of potential pathogens.

A spermicidal activity due to certain bacterial enzyme systems was observed by Marinov (1967). Part of this spermicidal activity was attributed to the elevated glucose dehydrase activity of E. coli, Staph. aureus or Bacillus prodigeosum. When any one of these organisms was added to semen 18-24 hours prior to insemination, conception rates declined.

Walsh, Hildebrandt and Prystowsky (1965) incubated species of vaginal bacteria in antibiotic-free human amniotic fluid. A significant increase in cell numbers was observed. In contrast, Galask and Snyder (1968) reported an increase in the lag phase together with growth inhibition in similar work with antibiotic-free human amniotic fluids.

The bacterial genera used in this study were Proteus sp., Pseudomonas sp., "coliforms," Staphylococcus sp. and Streptococcus sp.

The presence of steroid hormones in amniotic fluid was reported by Schindler and Suteri (1968) and Schindler and Ratanasapta (1968). A decrease in the amniotic fluid steroid hormone level was observed in human pregnancies following fetal distress or death. Osburn, Stabenfeldt and Ewing (1969) reported a sharp decrease in the plasma progesterone levels of cows prior to abortion.

V. fetus infection in the cow cannot be detected by clinical examination due to the innocuous nature of the disease. Its presence in a herd may be suspected when reductions of calf crops occur. Repeated returns of cows to service and mid term abortions are other signs of possible herd infection (Laing, 1960). Accurate diagnosis requires the isolation of the organism by cultural techniques (Lovell, 1964) or the demonstration of significant antibody titers in serum or vaginal-cervical mucus (Laing, 1960).

The true venereal vibriosis of cattle is caused by V. fetus var. venerealis. Another variety of V. fetus called V. fetus intestinalis causes some abortions in cattle but is primarily isolated from sheep. Florent (1959) first described these two isolants thus:

> V. fetus var. venerealis (Type I),
>
> H_2S negative, inhibited by 1% glycine or 1% sodium selenite with failure to grow on a selective medium containing brilliant green and bile salts.
>
> V. fetus var. intestinalis (Type II)
>
> H_2S positive, able to grow on 1% glycine and in 1% sodium selenite with subsequent reduction and with growth occurring on a selective medium containing brilliant green and bile salts.

Since certain isolants of V. fetus var. venerealis were later found to produce traces of H_2S, a subtype 1 classification was suggested by Florent (1963). The taxonomic position of V. fetus was challenged by Sebald and Veron (1963). These authors recommended the introduction of a new genus, Campylobacter, to accomodate V. fetus and the saprophytic V. bubulus.

Vibriosis is an asymptomatic infection in the bull. Samuelson and Winter (1966) studied the distribution of V. fetus var. venerealis in the preputial cavity and terminal urethra of the bull. The organism was found throughout the preputial cavity with the greatest number located in the preputial fornix and on the penis. The lumina of the epithelial crypts were considered as the major sites of proliferation. No lesions were observed. The authors were unable to detect the presence of local antibody. V. fetus var. venerealis was not considered an effective antigenic stimulus in the bull. Once established, the infection in the bull may persist for many years (Laing, 1960).

The infection in the heifer, or cow, is apparently confined solely to the reproductive tract and has been more widely studied than the infection in the bull. The infection was noted to be acute at first and later became chronic (Laing, 1960). A fresh isolant was found to persist in the uterus of the virgin heifer for a period of at least 13 weeks (Newsam and Peterson, 1964). The possibility of a loss of virulence was suspected by these workers when a laboratory adapted culture of V. fetus failed to presist when inoculated into the uterus of the virgin heifer. Vanderplassche, Florent and Huysman (1957) reported that V. fetus reached the uterus within five days after heifers

were bred to V. fetus infected bulls. The organisms were cleared from the non-pregnant uterus within three weeks. Vaginal cultures of infected cows were persistently positive for V. fetus for periods as long as eight months.

The role of V. fetus as a disease of the fetal membranes of cattle was early recognized by Smith (1918). From this work a series of papers (Smith, 1919; Smith, Little and Taylor, 1920; Smith, 1923) followed, in which the etiology of the infection and its role in bovine abortion was described. V. fetus infection was not found to persist with the tenacity shown by B. abortus (Smith et al., 1920). Cultures of fetal stomach contents, spleen, liver and lungs were positive for V. fetus. The experimental inoculation of pregnant cows resulted in abortion with isolations of V. fetus from the stomach contents of the aborted fetuses (Smith, 1919). The lesions described by Smith (1923) as the result of V. fetus infection included mushy, yellowish cotyledons, completely necrotic villi and edema of the chorion.

Smith (1918) stated that V. fetus showed a predilection for the caruncles of the uterus and produced abortion by destruction of the fetal-maternal vascular attachments. The evolution of a mild endometritis, within three weeks of the establishment of the infection, in the non-gravid uterus of the cow was described by Huysman (1957). It progressed from the superficial layer and reached the spongiosa within two months after the onset of the infection. The condition was found to heal from the deeper to the superficial layers and healing was apparently complete within three months from the onset of the infection. Similar lesions and healing patterns were reported by Frank, Shalkop, Bryner and O'Berry (1962). In addition to the endometrial changes

associated with a mild to moderate inflammatory reaction these authors observed a definite neutrophilic and lymphocytic infiltration of the endometrium. The relationship of the endometrial healing to the development of an immune response was reported by Dozsa, Mitchell and Olson (1962). Lymphocytosis was observed during the slow recovery of the uterus. This was accompanied by an increased resistance to a rechallenge inoculation with V. fetus. The new lesions produced by the second infection were less severe than those initially observed when the uterus first became infected. Peterson and Newsam (1964) described lymphocytic and plasmacytic reactions in the infected endometrium of the cow. No lesions were observed in the vagina. The presence of V. fetus in the reproductive tract produced no lesions at all in some of the experimental animals.

The occurrence of cervicitis and metritis as the result of intravenous or intracutaneous injection of V. fetus growth products into cows was demonstrated by Simon and McNutt (1957). Cervicitis and metritis was observed in the non-gravid reporductive tract of heifers in both naturally acquired and experimentally induced V. fetus infections. Osborne (1965) studied the effects of whole cell and cell-free supernatant fluids of V. fetus when injected intravenously into calves, pigs, goats, sheep and rabbits. Both reversible and irreversible bacterial shock resulted. Pregnancy was found to intensify the host reaction to the toxic principle. Hypersensitivity was suggested as a possible cause for abortion. A similar mechanism to explain V. fetus associated abortion was proposed by Manclark and Pickett (1965). Dennis (1959) isolated a toxic lipopolysacharide from V. fetus cells and demonstrated its abortive action on rabbits, mice, guinea pigs and sheep.

Osburn et al. (1969) reported embryonal death within five to 20 days after the inoculation of V. fetus into the uterus of the pregnant cow. Fetal death was followed by a decline in the plasma progesterone level. This decline in the hormone level preceded abortion and was attributed to placental dysfunction and luteolysis of the corpus luteum.

The immune response of the cow to V. fetus infection was reported by Manclark and Pickett (1965a). Cyclic variations of the cervical-vaginal mucosal antibody titers were observed. The highest antibody titers were determined at diestrus. Uterine infections with V. fetus led to the appearance of earlier and higher genital mucus antibody titers, which were greater than the antibody titers found in the serum.

Smith (1918) attempted to infect 40 guinea pigs with material containing viable V. fetus cells. The animals were inoculated via the subcutaneous and intraperitoneal routes. Cultures taken from the liver, spleen and kidneys of each animal five days later were negative for V. fetus. After this initial failure to recover the organisms, Smith and Taylor (1919) and Smith (1923) inoculated guinea pigs intraperitoneally with tissues from V. fetus infected cows, and successfully isolated V. fetus from the guinea pigs. Smith (1923) used three guinea pigs, which were inoculated at the same time but euthanized on days three, four and five post inoculation. Isolations of V. fetus were made from the spleen, liver and kidney tissues of those animals euthanized on days three and four post inoculation. Cultures taken from the animal examined on day five post inoculation were negative for V. fetus. Smith and co-workers (1918; 1919; 1920; 1923) made no reference to any gross

lesions in the guinea pigs studied. The use of the guinea pig in this early work was primarily to rule out the presence of B. abortus in the cattle which were being examined for causes of unexplained abortion.

Lerche (1937) induced abortion in guinea pigs by means of intraperitoneal, conjunctival and subcutaneous inoculation of viable V. fetus cultures. This author was unable to demonstrate V. fetus in the stomach contents of an aborted fetus.

Ristic and Morse (1953) experimentally induced V. fetus infection in the female guinea pig. A mixed inoculum of ovine and bovine isolants was used. Seven pregnant and three non-pregnant guinea pigs were inoculated intraperitoneally. V. fetus was either isolated by culture or demonstrated microscopically from all the pregnant animals, four of which aborted. The non-pregnant animals were negative on culture. Another group of guinea pigs was inoculated with B. abortus, USDA strain 19 in addition to V. fetus live cells. The pregnant animals were positive, while all but one of the non-pregnant animals were culturally negative

In a second experiment (Ristic and Morse, 1953), pregnant and non-pregnant guinea pigs were exposed to V. fetus by oral, subcutaneous and intravaginal routes of inoculation. All the non-pregnant animals were negative for V. fetus when cultured at necropsy 21-23 days post-inoculation. Two of nine pregnant animals aborted following oral inoculation and two of seven pregnant animals aborted following subcutaneous inoculation. Eleven pregnant animals delivered offspring after an uneventful gestation period. The intravaginal route of inoculation induced a single abortion. V. fetus was isolated from the embryo. The remainder of the animals inoculated intravaginally were negative for V. fetus when cultured 28 days after inoculation. It was proposed that

the guinea pig was a valuable animal in which to study the pathogenicity of V. fetus if the intraperitoneal route of inoculation was used to infect the animal.

Ristic, Wipf, Morse and McNutt (1954) described the lesions observed in the preceding work (Ristic and Morse, 1953). Epithelial sloughing, inflammatory cell reaction, hemorrhages, enlarged blood vessels, myometrial and subendometrial edema, together with cystic uterine glands were the major pathological changes listed. Histological changes most frequently observed in the fetal tissues were hemorrhages of the myocardium and liver. Black, Simon, McNutt and Casida (1953) were unable to detect any appreciable pathologic change in the estrous of pseudopregnant uterus or the rabbit after intrauterine inoculation with V. fetus.

Ristic, Morse, Wipf and McNutt (1954) were able to transmit V. fetus from infected female guinea pigs to non-infected female guinea pigs via coitus with a male as the carrier. In this study a sheep isolant, strain K, was used as the inoculum. An attempt was made to infect 10 females by this method and of these only two were found to be positive when euthanized 43 days after mating. Four of the 10 experimentally infected females gave positive cultures for V. fetus. This figure included two which aborted.

The use of the guinea pig as an animal for the diagnosis of V. fetus infection was proposed by Adler (1953). This author attempted to infect female guinea pigs by intravaginal inoculation during induced estrus. A single intramuscular injection of estradiol benzoate (250 IU) was given followed by another injection 72 hours later. Immediately after the second injection of hormone the guinea pigs were inoculated

intravaginally either with a pure V. fetus culture or with V. fetus contaminated semen. Six days later the animals were euthanized and cultures taken from the uterine horns. When pure cultures of a fresh isolant of V. fetus were inoculated, 10 of 11 animals were positive for V. fetus. An older, laboratory maintained stock culture of V.fetus failed to infect guinea pigs. When infected bovine semen was used as the inoculum, V. fetus was isolated from three of seven animals.

Further studies in the use of the female guinea pig as a diagnostic animal were reported by Power (1954). A group of six females was given diethylstilbestrol dipropionate, intramuscularly, to induce estrus. The animals were inoculated intravaginally with semen from a V. fetus infected bull. At necropsy, seven days post inoculation, V. fetus was isolated from three of the guinea pigs. Based on these results the author recommended the use of at least six animals if any attempts at diagnosis were contemplated.

V. fetus was isolated from infected bull semen by intraperitoneal inoculation into pregnant guinea pigs (Robinson, Van Rensburg, Van Heerden and Van Drimmelen, 1956). The animals were euthanized five to seven days after inoculation and the organism was recovered from the stomach contents of the fetuses. Robinson et al. (1956) reported failure in their attempts to infect two groups of stilbestrol treated female guinea pigs with a viable culture of V. fetus.

Manclark and Pickett (1965a) stated that the changes which occurred in the female guinea pig genital mucosa during the reproductive cycle compared favorably with those of the cow. From studies in which the guinea pig was used to observe the immune response to V. fetus infection, these authors concluded that V. fetus showed a predilection for the gravid

uterus, since pregnant or pseudopregnant guinea pigs were apparently susceptible to infection. The non-pregnant guinea pig was not susceptible. The pattern of antibody formation was dependent upon the route of inoculation. The inoculation of live cells by the intraperitoneal route led to the production of humoral antibody. The organisms then localized in the uterus and stimulated the production of mucosal antibody. Live antigen administered by the intravaginal route stimulated the production of local, but not circulating, antibody. This same pattern of immune response was found to occur in the cow.

The exact mechanism involved in the pathogenicity of V. fetus and how it effects reproductive efficiency is not fully understood. Bacterial species which produce infections of the female reproductive tract and thus have the potential to adversely affect pregnancy may be categorized into four major groups. 1) Those which produce endotoxins, i.e., Brucella sp., E. coli and V. fetus. 2) Organisms which disrupt the pattern of endometrial metabolism by the production of inflammatory reactions, i.e., Brucella sp. and V. fetus. 3) Organisms which directly infect the fetus, i.e., Leptospira and 4) those organisms which cause an imbalance of the uterine environment by the use of available metabolites in the lumen fluids. Such competition could affect the developing blastocyst before nidation.

V. fetus may apparently fall into all of the above listed categories. A study in which V. fetus var. venerealis is used to induce an infection in the female guinea pig may help to clarify the mechanism of pathogenesis of the organism on the reproductive tract and the embryo and, at the same time, permit a reevaluation of the guinea pig as a model in which to study the course of the infection.

MATERIALS AND METHODS

Principle characteristics of V. fetus var. venerealis used in this study

V. fetus var. venerealis is a Gram negative, motile, monoflagellated, slightly curved rod. It is catalase positive, H_2S negative and does not grow in media which contain 3.5% sodium chloride or 1.0% glycine. Growth occurs in 1.0% oxgall and in 0.1% sodium selenite. A gas mixture of 85% N_2, 10% CO_2 and 5% O_2 is required in which the organism grows at 25 C and 37 C but not at 42 C.

Source and maintenance of the isolants

The isolants of V. fetus var. venerealis were recovered from the reproductive tracts of cows slaughtered in Florida. These isolants were characterized and kindly made available to the author by Dr. F. H. White of the Department of Veterinary Science, University of Florida. After initial isolation and identification the isolants were preserved in the lyophilized state. To avoid repeated passage of the cultures after recovery from lyophilization, batches of 24-48 hour thioglycollate broth cultures were deep frozen at -80 C for subsequent use in the laboratory and animal studies.

Only smooth isolants were used throughout the study. Regular checks were made on the cultures to ensure the absence of dissociation. These checks were made by plate microscopic examination of bacterial colonies isolated on Brucella agar (Albimi).

Culture media

Basal media for growth. Thioglycollate broth (Difco) without dextrose or indicator was distributed in 10 ml volumes in 16x150 mm screw-cap culture tubes. Brucella broth with 0.16% added agar was similarly prepared. Brucella broth (Albimi) without added agar was distributed in 30 ml volumes to 50 ml capacity Ehrlenmeyer flasks and in 45 ml volumes to Nepheloflasks.* The media were sterilized at 121 C for 15 minutes

Solid media for the isolation of V. fetus and for viable cell counts. Selective bovine blood agar plates containing brilliant green and Novobiocin were prepared by the method of Lovell (1964). Brucella agar plates were prepared by the addition of 2.0% agar to Brucella broth (Albimi). The latter medium was sterilized at 121 C for 15 minutes.

Dilution blanks for viable cell counts

Deionized distilled water was added to screw-cap dilution bottles in 99.0 ml volumes. The water was sterilized at 121 C for 15 minutes.

Production of the required gaseous environment for the growth of Vibrio sp.

A large desiccator was used as a growth chamber. A desiccant of one part calcium chloride and three parts activated silica gel** was added to avoid excessive condensation of moisture. The growth chamber was used for the isolation of V. fetus on solid media or to propagate the organism in small flasks of Brucella broth without agar. The flasks or petri-dishes were placed in the desiccator and a negative pressure of 590 mm Hg

*Corning Glass Company, New York, N. Y.
**Tel-Tale, a product of Davison Chemical, Inc., Baltimore, Md.

was produced with a vacuum pump. CO_2 was used to decrease the negative pressure to 490 mm Hg. The negative pressure was then further reduced to 50 mm Hg by the addition of N_2. Each gas was delivered from a separate cylinder. The final atmosphere in the desiccator was approximately 10% CO_2, 85% N_2 and 5% O_2, the latter derived from the residual air. A partial negative pressure remained.

To grow the organism in volumes of fluid media greater than 30 ml and to allow the production of a gaseous environment in the Nepheloflasks, a commercially manufactured gas mixture* of 10% CO_2, 85% N_2 and 5% O_2 was used.

To sterilize the gas mixture a filter assembly was constructed in the laboratory. A large glass combustion tube, 100 cm x 4 cm, was packed with glass wool. Non-absorbent long fiber cotton was placed in each end at the top and bottom of the glass wool column. The ends were closed tightly with black rubber stoppers through which fire polished glass tubing, 0.5 cm outside diameter, was passed. One end of the filter carried a length of rubber tubing with the free end closed with a spring clip. The other end of the filter was left open. The entire assembly was wrapped in Kraft paper and sterilized at 121 C for 15 minutes.

To gas a fluid culture the filter was connected by the open end to the gas cylinder reducing valve. A sterile pipette was attached to the clamped rubber tubing and the clamp was released. After inoculation the flask was opened aseptically and the gas mixture passed into it with the tip of the pipette held just above the surface of the medium. The

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*Airco, Inc., Jacksonville, Fla.

flask was gassed for 30 seconds with the flow regulated at two pounds per square inch. The pipette was removed from the flask which was closed tightly with a sterile rubber stopper. A new sterile pipette was used for each flask gassed.

Hormones and organic compounds used in the in vitro growth experiments and for the regulation of the hormonal states of the guinea pigs

In vitro growth studies.

l-erythritol*
Follicule stimulating hormone (F.S.H.), porcine*
Estrone (1,3,5, (10)-estratrien-3-ol-17-one)*
Progesterone, U.S.P.**

Regulation of the hormonal states of the guinea pigs.

Progynon Benzoate*** (estradiol benzoate in oil 1 mgm per ml)
Progesterone solution (Repository type, 25 mgm per ml)****

Histological examination of tissues

All tissues were fixed in formalin, embedded in paraffin and sectioned at six microns. Sections were stained with hematoxylin and eosin stain.

Experimental animals

Mature, non-gravid female and mature male guinea pigs***** were used in mating studies and experiments in which the intravaginal route of inoculation was used. Ovariectomized and pregnant guinea pigs****** were used in studies which involved intrauterine inoculation. The animals were of mixed breed and English short-hair varieties. They were identified

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*Mann Research Laboratories, Inc., New York, N. Y.
**Matheson, Coleman and Bell, East Rutherford, N. J.
***Schering Corporation, Bloomfield, N. J.
****Norden Laboratories, Lincoln, Neb.
*****Kel Farm, Alachua, Fla.
******Camm Research Institute, Wayne, N. J.

by color and cage number. White animals were stained on the back to facilitate identification.

The animals were housed in stainless steel cages with one-half inch mesh floors, except for pregnant females in the late stages of gestation. These animals were housed on solid floors.

A diet of Purina guinea pig chow was fed, supplemented with fresh cabbage and hay twice weekly. Water was given ad libitum.

The animal quarters were maintained at a temperature of 68 F ± 5 F. Normal daylight was provided in the animal room. Male animals were housed in separate facilities but under the same conditions as the females. Females were taken to the males for breeding experiments. Mating was polygamous with two to three females to each male.

Determination of female estrus cycles. Estrus cycle lengths were determined by examination of the vaginal cytology (Asdell, 1946). Daily vaginal smears were taken with a small blunt-ended dropper fitted with a rubber bulb. A small volume of 0.85% saline was washed into the vagina and withdrawn. The material collected was transferred to a clean glass slide and allowed to air dry. The smears were stained with a modified Wright Giemsa stain technique. Wright stain was applied to the smear for one minute to effect fixation. The Wright stain was diluted, 1:4, on the slide with Wright buffer and the smear stained for six minutes. The smears were washed with distilled water and the excess water drained off. The smears were next stained with a dilution of 1:50 Giemsa stain and Wright buffer for 1 1/2 hours. Finally the smears were rinsed in distilled water and air dried. The smears were examined microscopically at magnifications of X450 and X950. Cytological data for each animal were collected over a period of 20

days and recorded. The animals were then placed into proestrus, luteal and follicular phase groups. The mean cycle length was 16 ± 2 days.

In vitro growth studies V. fetus var. venerealis

Experiment 1. Erythritol as a growth factor. Stock solutions of erythritol were prepared. Solution 1 contained 2 gm of erythritol in 4 ml of distilled water (W/V). Solution 2 contained 0.3 gm erythritol in 10 ml of distilled water (W/V). These solutions were sterilized by passage through a 0.22 pore size membrane filter.* Volumes of Brucella broth (Albimi) were prepared in duplicate Nephelo-flasks so that the final volume of liquid medium after the addition of the inoculum and erythritol solution was 50 ml. The broth was added to the flasks before sterilization and the erythritol was added aseptically after the medium was cooled. The flasks of medium were prepared as follows.

Flask No.	ml of stock solution and solution No.	ml of Brucella broth	ml of inoculum	Final conc erythritol per ml
1 and 2	0.1, sol 2	44.9	5.0	0.06 mgm
2 and 3	0.1, sol 1	44.9	5.0	1.0 mgm
4 and 5	0.3, sol 1	44.7	5.0	3.0 mgm
5 and 6	0.5, sol 1	44.5	5.0	5.0 mgm
7 and 8	1.0, sol 1	44.0	5.0	10.0 mgm
9 and 10	control	45.0	5.0	-

Seed cultures of V. fetus were prepared in 30 ml of Brucella broth without added agar. These cultures were placed in the gas jar and incubated at 37 C on a New Brunswick Model G incubator shaker at 100 oscillations per minute. After an incubation period of 36 hours the

*Millipore Filter Corporation, Bedford, Mass.

seed culture was checked for motility, morphology and purity. Synchronization of the culture was effected by passage through five layers of sterile Whatman number 42 filter paper under aseptic conditions.

An inoculum of 5 ml synchronized cells suspended in Brucella broth was added to the test and control Nepheloflasks. These were gassed and stoppered tightly with black rubber stoppers. The optical density (OD) of each flask was read at time zero on a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 525 mμ. Brucella broth was used as a colorimeter blank. The Nepheloflasks were incubated on the shaker at 37 C and shaken at 100 oscillations per minute. The OD of each flask was read at intervals between eight to 12 hours until the exponential growth phase of the cultures was completed. A final check of cell morphology was made at the end of the experiment.

Graphs of OD against time were plotted for each test dilution and control. The slopes of these graphs were determined. The data were examined for significant differences between the test and control growth curves by an analysis of variance and Duncan's Multiple Range Test (Steel and Torrie, 1960).

Experiment 2. Estrone as a growth factor. A stock solution of estrone was prepared by the addition of 15 ml of analytical grade chloroform (99.9% pure) to 0.06 gm of estrone. Nepheloflasks which contained 35 ml of Brucella broth, without added agar, were sterilized at 121 C for 15 minutes and allowed to cool. Stock estrone solution was added aseptically to the flasks as follows.

Flask No.	Volume of estrone solution ml	ml of inoculum	final conc of estrone %
1 and 2	1.0	5.0	0.01
3 and 4	0.9	5.0	0.009
5 and 6	0.7	5.0	0.007
7 and 8	0.5	5.0	0.005
9 and 10	0.3	5.0	0.003
11 and 12	0.1	5.0	0.001
13 and 14	0.05	5.0	0.0005
Controls (2)	-	5.0	-

A series of reagent blanks were prepared by the addition of estrone in the above volumes to Nepheloflasks which contained 40 ml of sterile Brucella broth but were not inoculated with V. fetus. The chloroform was removed from the test and reagent blank flasks by gentle steam heat applied to the outside of the flasks. The hormone precipitated in the broth as the chloroform was removed. The test and control flasks were inoculated with 5 ml of a 36-hour synchronized seed culture of V. fetus. Each was gassed and tightly stoppered.

An initial OD was read at time zero against a blank of Brucella broth. The optical densities of the test hormone dilutions were corrected by the subtraction of the reagent blank OD from its respective test flask OD. The flasks were incubated on the gyrotary shaker at 37 C. The OD was determined at regular intervals throughout the exponential growth phase of the organism.

A growth control to determine the effects of the chloroform on the growth of V. fetus was prepared by the addition of chloroform to Brucella broth followed by its removal by gentle steam heat and inoculation with 5 ml of the synchronized culture. This control was incubated under the same conditions as the tests and non-chloroformed controls. The results of both controls were subsequentely compared (see Appendix A).

Data collection, statistical analyses and morphological checks of the cultures were identical to those described in Experiment 1.

Experiment 3. Progesterone as a growth factor. A stock solution of progesterone was prepared by the addition of 15 ml of analytical grade chloroform to 0.06 gm of progesterone. The test was performed in accordance with the procedure described in Experiment 2.

Experiment 4. Estrone and progesterone as combined growth factors. Nepheloflasks of 35 ml of Brucella broth without added agar were sterilized at 121 C for 15 minutes. Stock solutions of estrone and progesterone were prepared (see Experiments 2 and 3 above). These were added to the flasks of broth in the following manner.

Flask No.	Vol. Estrone stock sol ml	Vol. Progesterone stock sol ml	Vol. of inoculum ml	Final estrone progesterone conc %
1, 2 and 3	0.1	0.9	5.0	0.001/0.009
4, 5 and 6	0.3	0.7	5.0	0.003/0.007
7, 8 and 9	0.5	0.5	5.0	0.005/0.005
10, 11 and 12	0.7	0.3	5.0	0.007/0.003
13, 14 and 15	0.9	0.1	5.0	0.009/0.001
Controls	-	-	5.0	- -

Control cultures, reagent blanks and colorimeter blanks were prepared by the methods described in Experiment 2. Inoculation, incubation and data collection and examination were similarly performed.

Experiment 5. Follicule Stimulating Hormone (FSH) as a growth factor. Nepheloflasks of Brucella broth (w/o agar) were sterilized at 121 C for 15 minutes. A vial of FSH (porcine) which contained 50 mgm of hormone was reconstituted with 10 ml of sterile distilled water. A series of dilutions of FSH in broth was prepared in the cooled medium

as follows.

Flask No.	Stock FSH sol ml	Volume of broth ml	Vol. of inoculum ml	Final FSH conc %
1, 2 and 3	0.8	34.20	5.0	0.01
4, 5 and 6	0.4	34.60	5.0	0.005
7, 8 and 9	0.24	34.76	5.0	0.003
10, 11 and 12	0.08	34.92	5.0	0.001
13, 14 and 15	0.04	34.96	5.0	0.0005
Controls	-	35.00	5.0	-

Test and control flasks were inoculated with 5 ml of a 36-hour synchronized culture, gassed and tightly stoppered. Incubation, data collection and culture checks were performed by the methods described in Experiment 1. A statistical analysis of the data was also made by the methods outlined in Experiment 1.

V. fetus var. venerealis infection of the non-gravid guinea pig

Experiment 6. Intravaginal inoculation during normal follicular and luteal reproductive cycle phases. Two groups of three virgin female guinea pigs each were selected on the basis of vaginal cytology into follicular and luteal phase groups. All the animals were mature and weighed between 450-500 gm. A control group comprised two animals, one in the follicular phase of the reproductive cycle and the other in the luteal phase. Preliminary vaginal cultures of all the animals were plated to selective blood agar plates which were incubated in the gas mixture at 37 C.

A culture of V. fetus was prepared in Brucella broth for use as the inoculum. A check of motility, morphology and cultural characteristics was made before the inoculation procedure was undertaken. A viable cell count was performed on the broth culture.

One ml of the broth culture for each inoculum required was

was centrifuged at 3000 G to concentrate the cells. The supernatant fluid was decanted and the cell pellet resuspended in 0.3 ml of Brucella broth. The animals in each test group were inoculated intravaginally on each of three days with 0.3 ml of concentrated cell suspension (approximately 1×10^7) cells. The inoculum was placed in the vagina with a sterile glass serological pipette fitted with a rubber bulb. The control animals were inoculated with 0.3 ml of cell free Brucella broth over the same time period.

Before the administration of the inoculum on the second and third days of the inoculation period, a vaginal culture and smear for Gram stain was taken from each test and control animal. Vaginal cultures and smears were also taken from the test and control animals for three consecutive days following the final inoculation.

Five days after the final inoculation each test and control animal was euthanized. The entire reproductive tract was aseptically removed and placed in a sterile petri dish. Cultures were taken from the vagina, proximal and distal cervical area, both uterine horns, both ovaries, liver, spleen and blood from the heart. These were plated on selective blood agar plates and into thioglycollate broth. Portions of the uterine horns were opened with sterile scissors and placed into thioglycollate broth without dextrose or indicator. Vaginal and cervical cultures were plated to selective blood agar plates and to thioglycollate medium. The ovaries were cultured in thioglycollate broth. Any organisms which grew in the thioglycollate broth cultures were subcultured to selective blood agar. All solid mediums were incubated at 37 C in the gas mixture. Uterine and cervical tissues were preserved in 10% formalin for histological examination.

Experiment 7. Intravaginal inoculation of the normal estrus guinea pig. Twenty-four mature virgin female guinea pigs between 500-600 gm in weight were divided into a test group and a control group with 12 animals in each group. A preliminary vaginal culture was made on each animal to determine a vibrio free status (see Experiment 6). Vaginal cytological examinations were performed and each test animal in the latter stages of the follicular phase of the cycle was inoculated with approximately 3×10^7 viable cells daily until estrus was observed. Control animals at the same phase of the reproductive cycle were inoculated with sterile Brucella broth. The inoculum was prepared and given by the methods described in Experiment 6.

Both test and control animals were mated to fertile males in the ratio of two or three females to a single male. The females were examined weekly for pregnancy by gentle abdominal palpation and removed from the male as soon as signs of pregnancy were observed. All pregnancies which resulted were allowed to continue to term. Maternal or fetal deaths, stillbirths or other complications of pregnancy were investigated by necropsy and extensive cultural examination. In the case of maternal death in the test group, the stomach contents of all the embryos *in utero* were cultured in thioglycollate broth. The abdominal and thoracic viscera of the embryos were similarly cultured. Selective bacteriological examination of the fetal-maternal membranes and reproductive tract of the dam was made for *V. fetus*. In addition, routine bacteriological culture techniques were used to enable the isolation of other potentially pathogenic organisms. Stillborn offspring and offspring which died within 48 hours of birth were necropsied

and the stomach contents cultured for V. fetus. Routine bacteriological examination was also made of the liver and spleen. In cases of maternal death, histological examinations were made of the fetal-maternal membranes and the uterus of the dam. Any complications of pregnancy which occurred in the control animals were similarly investigated.

The reproductive capability of the test and control animals was examined for significant difference by the Student's "t" test (Steel and Torrie, 1960).

Experiment 8. Intravaginal inoculation during induced estrus. Six virgin female guinea pigs weighing between 500-600 gm were used as test animals. Two similar animals were exogenous hormone-free controls. The vibrio-free status of all the animals was confirmed by vaginal cultures which were plated on selective blood agar and inoculated into thioglycollate broth.

All the test animals received two intramuscular injections of 300 IU estradiol benzoate, the second injection 72 hours after the first. Vaginal smears for cytological study were taken at the time of the second injection to determine the presence of induced estrus. Cytological studies were also made on vaginal smears of the control animals to detect proestrus conditions in their reproductive tracts.

Immediately after the second injection of hormone an inoculum of approximately 3×10^5 viable cells was given intravaginally to both test and control animals. The inoculation was repeated after 24 and 48 hours. Vaginal smears for Gram stain and vaginal cultures were taken at the same time intervals described in Experiment 6.

Five days after the final inoculation the animals were euthanized. Necropsy, bacteriological and histological examinations of test and control animals were made by previously described methods (see Experiment 6).

Experiment 9. Intravaginal inoculation of progesterone treated females. Six virgin female guinea pigs weighing between 500-600 gm were used as test animals. The control group comprised two similar animals. Each test animal was given 2.5 mgm of progesterone intramuscularly daily for three days. Preliminary vaginal cultures were taken on all the animals to confirm a vibrio-free status. A check of vaginal cytology was made to ensure the effectiveness of the exogenous hormone treatment.

After the third hormone injection each test animal was inoculated intravaginally with an inoculum of approximately 3×10^6 viable cells. The control animals were similarly inoculated. The inoculation was repeated at 24 and 48 hours. The study was completed by the methods employed in Experiment 6.

Intrauterine inoculation of ovariectomized guinea pigs treated with exogenous hormones

Experiment 10. Inoculation during exogenous estrogen administration. Twelve ovariectomized guinea pigs of 500-550 gm weight were given a single intramuscular injection of estradiol benzoate (1000 IU). The animals were prepared for surgery 72 hours after the hormone injection. The hair coat was clipped and shaved from the abdomen and solid food was withheld for a 24-hour period. Anesthesia was induced with methoxyfluorane* and the animal placed in a position

*Metofane, a product of Pitman-Moore, Dow Chemical Co., Indianapolis, Ind.

of dorsal recumbancy on a small laboratory animal board.* A 3.0 cm incision was made in the abdominal wall and the uterus exposed through the incision. An inoculum of 1.2×10^6 viable cells of V. fetus contained in 0.2 ml of Brucella broth was introduced into the right horn of the uterus midway between the point of bifurcation and the fallopian tube. The inoculum was delivered from a tuberculin syringe fitted with a 27-gauge needle. The left horn of the uterus was injected with 0.3 ml of sterile Brucella broth for use as a control. The incision was closed with 000 gut and surgical clips. The animals were observed until recovery from anesthesia was complete.

The guinea pigs were euthanized in groups of four at 24, 48 and 72 hour intervals post-surgery. A necropsy was carried out under aseptic conditions. The viscera were examined for gross lesions and any fluid in the peritoneal cavity was cultured in thioglycollate broth. The entire reproductive tract was removed aseptically. A ligature was tied around the right uterine horn at its junction with the fallopian tube to facilitate orientation of the tract after its removal. The two uterine horns were dissected free from the cervix and the fallopian tubes. Both horns were flushed with 5 ml of sterile phosphate buffered saline (pH 7.2) and the washings collected into sterile centrifuge tubes. A viable cell count was performed on 1 ml of the saline washings from the right horn. The 4 ml volume which remained was centrifuged at 3000 g and the sediment cultured on selective blood agar plates and in five tubes of thioglycollate broth. Smears were also prepared for Gram stain and cytological study. Transverse

*Germfree Laboratories, Inc., Miami, Fla.

sections of both uterine horns were prepared for histopathological study.

The cervix was cultured on selective blood agar plates and in thioglycollate broth. Transverse sections were taken for histopathological study. The vagina was similarly cultured but was not histologically examined. The liver, spleen and blood from the heart of each animal were cultured for V. fetus. In addition, cultures were taken from the liver, spleen, vagina, uterus, cervix and peritoneum for organisms other than V. fetus. These were plated to bovine blood agar plates and Eosinmethylene blue medium and incubated at 37 C.

Experiment 11. Inoculation during exogenous progesterone administration. Twelve ovariectomized guinea pigs weighing 500-550 gm were primed with 50 IU of estradiol benzoate given intramuscularly. Twenty-four hours later an intramuscular injection of 2.5 mgm progesterone was given. This was repeated 48 hours later. Twenty-four hours after the second injection of progesterone the animals were taken to surgery.

Preoperative preparation, operative technique, inoculation and subsequent cultural and histological examinations were identical to those employed in the exogenous estrogen study (see Experiment 10).

Exposure of the gravid guinea pig to V. fetus

Experiment 12. Intrauterine inoculation of guinea pigs. Thirteen pregnant guinea pigs in the 30th day of gestation were separately housed and identified. An inoculum of 3×10^6 viable cells in 0.2 ml of Brucella broth was prepared (see Experiment 6). Eleven of the animals were inoculated directly into the right uterine horn with 0.2 ml

of the whole broth culture. The inoculum was placed in the amnionic cavity but directed away from the embryo. The control animals were sham operated and inoculated in a similar manner with 0.2 ml of sterile Brucella broth. The surgical technique employed was described in Experiment 10.

Two animals which aborted within 24 hours after inoculation were euthanized. A necropsy was performed under aseptic conditions and tissues from the reproductive tract, placentae, liver and spleen of the dams were cultured for V. fetus and other potential pathogens. The thoracic and abdominal viscera of all fetuses, whether aborted or in utero, were cultured in thioglycollate broth. If the stomach of an embryo contained fluid this was cultured separately in thioglycollate broth. Tissues from both the dams and the aborted fetuses were preserved for histological examination. A single animal which aborted 48 hours after inoculation was euthanized and cultured aseptically at necropsy. Tissues were saved for histological examination.

Two animals were euthanized 72 hours after inoculation and bacteriological and histological examinations were made. Two animals which did not abort after inoculation were allowed to go to term. The animals which aborted after inoculation and were not euthanized were cultured daily from the vagina until negative cultures for V. fetus were obtained.

RESULTS

In vitro studies of V. fetus var. venerealis

Experiment 1. Erythritol as a growth factor. The recorded optical densities and mean changes in OD per unit time of the V. fetus cultures are given in Table 1. The slopes of the curves derived during the unit time interval are given in Appendix A. The analysis of variance of these slopes gave a calculated F value of 4.11. The tabulated F values at the 0.01 and 0.05 levels of significance were given as 8.75 and 4.39 respectively. No significant differences were found between the test and control slopes. The organisms maintained their typical curved morphology throughout the experiment and no apparent change in the size of the cells was observed.

Experiment 2. Estrone as a growth factor. The recorded optical densities and mean changes in OD per unit time of the V. fetus cultures are given in Table 2. The slopes of the curves derived during the unit time interval are given in Appendix A. The analysis of variance of these slopes gave a calculated F. value of 3.32. The tabulated F values at the 0.01 and 0.05 levels of significance were given as 6.18 and 3.50 respectively. No significant differences were found between the test and control slopes (see Appendix A). There was no apparent change in the morphology or size of the cells during the experiment.

Table 1. Optical density readings of *V. fetus* cultures with erythritol added.

Time hours	Erythritol concentrations (mgm per ml) 0.06 A	0.06 B	1.00 A	1.00 B	3.00 A	3.00 B	5.00 A	5.00 B	10.00 A	10.00 B	Controls A	Controls B
0	.032	.040	.040	.030	.028	.029	.040	.045	.030	.030	.030	.032
[8	.130	.130	.142	.140	.128	.135	.140	.140	.120	.120	.140	.145]
16	.260	.245	.315	.345	.300	.355	.330	.300	.300	.330	.330	.350
[24	.450	.405	.530	.470	.508	.470	.470	.560	.500	.470	.550	.508]
32	.560	.540	.530	.470	.520	.470	.470	.600	.510	.460	.600	.500
44	.600	.590	.590	.450	.500	.440	.430	.610	.460	.420	.700	.470
*	.297		.359		.357		.375		.360		.386	

A and B are replicates of each erythritol concentration.

*Mean change in OD in 16 hours determined between [8 and 24] hours.

OD readings taken at 525 mμ .

Table 2. Optical density readings of V. fetus cultures with estrone added.

Time hours	Estrone concentrations (%)															
	0.01		0.009		0.007		0.005		0.003		0.001		0.0005		Controls	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	.120	.120	.120	.120	.120	.120	.120	.120	.120	.120	.120	.120	.130	.120	.120	.120
[3	.180	.170	.180	.180	.170	.170	.180	.180	.170	.170	.170	.170	.160	.180	.160	.160]
5	.240	.240	.240	.240	.210	.220	.220	.220	.220	.230	.220	.230	.230	.230	.220	.220
[7	.310	.310	.300	.300	.250	.290	.280	.280	.280	.280	.280	.280	.290	.290	.270	.280]
9]	.360	.340	.350	.350	.300	.330	.330	.340	.330	.330	.330	.320	.340	.330	.310	.300]
11	.380	.360	.370	.360	.320	.380	.370	.370	.360	.360	.360	.360	.370	.350	.350	.350
16	.460	.440	.450	.444	.380	.450	.450	.430	.430	.420	.430	.430	.440	.440	.440	.460
20	.510	.490	.480	.470	.430	.470	.490	.480	.510	.500	.500	.480	.510	.490	.510	.500
24	.490	.490	.480	.490	.420	.490	.490	.500	.510	.500	.500	.480	.510	.500	.520	.520
*	.175		.170		.145		.155		.160		.155		.165		.145	

A and B are replicates of each estrone concentration.

*Mean change in OD in six hours determined between [3 and 9] hours.

OD readings taken at 525 mμ.

Experiment 3. Progesterone as a growth factor. The recorded optical densities and mean changes in OD per unit time of the V. fetus cultures are given in Table 3. The slopes of the curves derived during the unit time interval are given in Appendix A. The analysis of variance of these slopes gave a calculated F value of 2.53. The tabulated F values at the 0.01 and 0.05 levels of significance were given as 6.37 and 3.50 respectively. No significant differences were found between the test and control slopes (see Appendix A). The organisms maintained their typical curved morphology throughout the experiment and no apparent change in the size of the cells was observed.

Experiment 4. Estrone and progesterone as combined growth factors. The recorded optical densities and mean changes in OD per unit time of the V. fetus cultures are given in Table 4. The slopes of the curves derived during the unit time interval are given in Appendix A. The analysis of variance of these slopes gave a calculated F. value of 8.76. The tabulated F values at the 0.01 and 0.05 levels of significance were given as 5.06 and 3.11 respectively. Significant differences were found between the slopes. Duncan's Multiple Range Test when applied to the data at the 5% level of significance indicated that the slopes of test concentrations of 0.005/0.005, 0.007/0.003 and 0.009/0.001% estrone/progesterone were significantly greater than the slopes of the hormone-free controls but were not significantly different from each other. The slopes of test concentrations of 0.005/0.005 and 0.009/0.001% estrone/progestrone were significantly greater than the slopes of test concentrations of 0.001/0.009 and 0.003/0.007% estrone/progesterone (see Appendix A). The organisms underwent no apparent changes in size or morphology

Table 3. Optical density readings of V. fetus cultures with progesterone added.

Time hours	Progesterone concentrations (%)															
	0.01		0.009		0.007		0.005		0.003		0.001		0.0005		Controls	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	.220	.220	.210	.210	.210	.210	.210	.210	.200	.210	.220	.215	.180	.200	.220	.220
[2	.280	.280	.270	.270	.280	.290	.250	.255	.250	.250	.230	.225	.240	.245	.240	.240]
4	.320	.320	.350	.340	.320	.330	.285	.280	.270	.275	.270	.280	.280	.270	.320	.280
6	.365	.360	.370	.350	.370	.360	.310	.325	.300	.300	.310	.315	.315	.300	.350	.310
[8	.410	.400	.410	.390	.400	.420	.350	.345	.340	.345	.360	.360	.340	.330	.390	.370]
10	.430	.425	.450	.420	.430	.430	.400	.400	.375	.365	.400	.415	.385	.380	.425	.390
12	.500	.450	.510	.440	.440	.450	.420	.415	.390	.410	.400	.410	.410	.400	.460	.420
*	.125		.130		.125		.095		.092		.132		.092		.140	

A and B are replicates of each progesterone concentration.

*Mean change in OD in six hours determined between [2 and 8] hours.

OD readings taken at 525 mμ.

Table 4. Optical density readings of V. fetus cultures with estrone and progesterone added.

	Estrone and progesterone concentrations (%)																	
	0.001/0.009			0.003/0.007			0.005/0.005			0.007/0.003			0.009/0.001			Controls		
Time hours	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
0	.190	.240	.320	.310	.240	.260	.230	.220	.230	.160	.170	.160	.210	.200	.210	.120	.130	.130
[2	.290	.360	.290	.350	.330	.340	.310	.300	.300	.250	.260	.260	.290	.290	.290	.190	.200	.210]
4	.350	.410	.360	.420	.400	.400	.390	.370	.370	.310	.340	.340	.360	.350	.370	.250	.260	.270
[6	.390	.460	.420	.470	.450	.460	.460	.440	.440	.380	.400	.380	.440	.420	.450	.300	.300	.310]
8	.380	.470	.380	.490	.470	.480	.450	.440	.430	.400	.400	.400	.450	.420	.440	.330	.320	.330
10	.420	.520	.470	.510	.490	.490	.500	.470	.470	.430	.440	.450	.480	.480	.510	.350	.370	.370
*	.110			.120			.143			.130			.146			.103		

A, B and C are replicates of each estrone/progesterone concentration.

*Mean change in OD in four hours determined between [2 and 6] hours.

OD readings taken at 525 mμ .

during the experiment.

Experiment 5. Follicle stimulating hormone as a growth factor. The recorded optical densities and mean changes in OD per unit time of the V. fetus cultures are given in Table 5. The slopes of the curves derived during the unit time interval are given in Appendix A. The analysis of variance of these slopes gave a calculated F value of 2.42. The tabulated F values at the 0.01 and 0.05 levels of significance were given as 5.06 and 3.11 respectively. No significant differences were found between the test and control slopes (see Appendix A). There was no apparent change in the morphology or size of the cells during the experiment.

The effect of chloroform treated medium on the growth of V. fetus. The means of the slopes of growth curves derived from V. fetus cultures grown in Brucella broth were compared to the means of the slopes of growth curves of V. fetus grown in chloroform treated Brucella broth. The Student's "t" test gave a calculated value of less than 1. No significant difference was found between the two growth curve means (see Appendix A).

In vivo studies of the non-gravid guinea pig

Experiment 6. Intravaginal inoculation during the normal follicular and luteal phases of the reproductive cycle. All the test and control animals were free of V. fetus at the beginning of the experiment. The test animals showed no apparent physiological response to the inoculum. Vaginal discharge was absent and the animals remained alert and active. No anorexia was observed.

Microscopic examination of Gram and Wright-Giemsa stained vaginal

Table 5. Optical density readings of V. fetus cultures with FSH added.

Time hours	FSH concentrations (%) 0.01			0.005			0.003			0.001			0.0005			Controls		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
0	.160	.170	.200	.170	.170	.170	.170	.170	.180	.170	.160	.170	.170	.160	.170	.180	.180	.180
[2	.240	.250	.270	.240	.240	.260	.250	.250	.250	.240	.250	.250	.240	.220	.240	.250	.250	.250]
4]	.340	.340	.350	.330	.330	.320	.330	.340	.340	.330	.340	.340	.330	.310	.330	.350	.340	.340
6	.370	.390	.420	.370	.380	.400	.390	.380	.380	.380	.400	.380	.370	.350	.380	.410	.400	.390
*	.090			.080			.080			.090			.090			.094		

A, B and C are replicates of each FSH concentration.

*Mean change in OD in two hours determined between [2 and 4] hours.

OD readings taken at 525 mμ.

smears prepared during the inoculation period showed some neutrophils together with degenerating epithelial cells and cell debris. The vaginal flora was comprised of Gram positive and Gram negative cocci and bacilli. Organisms morphologically similar to V. fetus were observed in two of six animals during the inoculation period but at no time during the post inoculation period or at necropsy five days later. There was apparently a slight increase in the number of leukocytes seen on the third day post inoculation but the increase did not become pronounced.

On each day of the three-day inoculation period V. fetus was isolated from the vaginal cultures of the test animals. Isolation of V. fetus was made from vaginal cultures taken from all the test animals 24 hours after the final inoculation was given. Positive vaginal cultures were obtained from four of six test animals 48 hours after the final inoculation was given and from two of six after 72 hours. No isolations of V. fetus were made from the vaginal cultures of the test animals at necropsy five days after the final inoculation was given (see Table 6).

The uterus, cervix, blood, liver and spleen of each test animal was cultured at necropsy and V. fetus was not isolated. Cultures of the left and right uterine horns were free from secondary or contaminating flora as were those of the liver and spleen. Pseudomonas aeruginosa, E. coli, Streptococcus sp. and Neisseria sp. were isolated from the vaginas of the test and control animals. All the cultures and smears taken from the control animals were negative for V. fetus.

No gross lesions were observed at necropsy. Histological studies of the uterine tissues revealed a slight inflammatory reaction

Table 6. Vaginal cultures of guinea pigs during and after intravaginal inoculation with V. fetus.

Phase of cycle	Animal number	During inoculation* 24 hrs.	48 hrs.	Post inoculation 24 hrs.	48 hrs.	72 hrs.	Necropsy 5 days post inoculation**
	1	+	+	+	+	+	-
Follicular	2	+	+	+	-	-	-
	3	+	+	+	+	-	-
Total		3/3	3/3	3/3	2/3	1/3	0/3
	4	+	+	+	+	-	-
Luteal	5	+	+	+	-	+	-
	6	+	+	+	+	-	-
Total		3/3	3/3	3/3	2/3	1/3	0/3
Cycle control animals							
Follicular	7	-	-	-	-	-	-
Luteal	8	-	-	-	-	-	-

+ = growth of V. fetus ; - = no growth of V. fetus in 5 days at 37 C.
* Cultures taken at 24 and 48 hous after day 1 of the inoculation period.
** No isolations of V. fetus were made from the uterus, cervix, blood, liver or spleen.

and congestion of the lamina propria with evidence of edema between the cells. No real differences were observed between the follicular or luteal phase animals which could be attributed to the inoculation of V. fetus.

Experiment 7. Intravaginal inoculation of the normal estrous guinea pig with subsequent mating. The statistical analysis of the results of this experiment showed a significant difference ($p = 0.05$) between the average number of young which survived in the control population when compared with that of young which survived in the test population. The average for the controls was 2.8 and that for the test animals was 1.3 (see Appendix A). The average number of young carried by the control group was 3.3. The average of the test group was 2.3. This difference was significant ($p = 0.05$). Although the litter size of the control animals was greater than that of the test group, the number of stillbirths in the control group was higher. One animal (I) in the control population and two in the test population (9 and 10) failed to show definite signs of pregnancy. These results are presented in Table 7.

A single animal (2) in the test group died in late gestation and four mummified fetuses were found in the uterus at necropsy (see Appendix C). Bacteriological examinations of the fetal and maternal tissues were negative for V. fetus. Histopathological examination of the tissues from the reproductive tract of the dam revealed thrombosis and extreme congestion of the endometrial vessels and some sloughing of the endometrium. Extreme necrosis of the placenta was noted especially around the area of attachment to the

Table 7. Reproduction efficiency of guinea pigs after intravaginal inoculation with V. fetus followed by natural mating.

	Animal identification	Size of litter	Number stillborn	Number surviving more than 30 hrs. post-partum	
Test group	1	2	0	0	
	2	4	0	0	Died; 4 mummified fetuses
	3	3	1	0	
	4	4	1	3	
	5	3	0	3	
	6	3	0	3	
	7	4	0	4	
	8	ND	ND	ND	V. fetus isolated from vagina
	9	0	0	0	Nonpregnant
	10	0	0	0	Nonpregnant
Total		23	2	13	
Control group	A	3	0	0	
	B	5	1	4	
	C	4	1	3	
	D	5	0	5	
	E	3	0	3	
	F	3	1	2	
	G	4	2	2	
	H	1	0	1	
	I	0	0	0	Nonpregnant
	J	5	0	5	
Total		33	5	28	

ND = not determined; animal aborted.

uterine wall. No nuclear staining was present. The liver showed some evidence of fatty change.

Three weeks after intravaginal inoculation one animal (8) in the test population was observed to have a bloody vaginal discharge which was characterized by increased numbers of neutrophils, numerous erythrocytes and contained many vibrio-like organisms. Subsequent isolation of V. fetus was made from a vaginal culture. No apparent abortion was observed; however, the animal failed to show any obvious signs of pregnancy and when euthanized six days after the expected date of delivery was found non-pregnant. No gross lesions were observed in the reproductive tract of this animal and no isolations of V. fetus were made from the reproductive tract, liver, spleen, blood or peritoneum.

The offspring of test animals No. 4, 5, 6 and 7 were apparently normal. The offspring of test animals 1 and 3 showed signs of extreme weakness immediately after birth and did not attempt to nurse the dams. Their gait was characterized by muscular twitching and staggering. All died within 30 hours post-partum.

Experiment 8. Intravaginal inoculation during induced estrus. All the test and control animals were free of V. fetus at the beginning of the experiment. The animals tolerated the estrogen injections well and a physiological response to the hormone was observed. The vagina dilated and there was an increase in the amount of vaginal mucus, which was profuse in one of the test animals. The inoculum of bacteria was tolerated well by both test and control animals. They remained active and alert throughout the test period. No anorexia was observed.

At the time of inoculation the vaginal cytology of the test and control groups was characterized by cornified epithelial cells and some cellular debris. The results of vaginal cultures taken during the test period are given in Table 8. The vaginal cultures of the test and control animals were positive for V. fetus during the inoculation period but became negative within 24 hours after the final inoculation. No isolations of V. fetus were made from vaginal cultures taken from the test or control guinea pigs at intervals of 48 and 72 hours after the final inoculation was given.

Cultures for V. fetus taken from the uterus, cervix, blood, liver and spleen of the test and control animals at necropsy five days after the end of the inoculation period were negative.

Gram stained preparations of vaginal smears of test and control animals taken during the inoculation period showed organisms morphologically similar to V. fetus in only one out of six animals. A mixed vaginal flora of Gram positive and Gram negative bacilli and cocci was observed. These secondary organisms when cultured included Proteus sp., E. coli, Streptococcus sp., Staphylococcus sp., Neisseria sp. and Diphtheroid organisms. The uterine horns of all the animals were apparently free of secondary microorganisms.

Cytological studies of the vagina of the estrogen treated animals made on the second day after the final inoculation showed a marked increase in the number of neutrophils accompanied by a decrease in the total number of microorganisms observed.

Histological examination showed no abnormalities in the uterine tissue of any of the animals. The condition of the endometrium and uterine glands was compatible with that of the uterus at estrus.

Table 8. Vaginal cultures of estrus induced guinea pigs during and after intravaginal inoculation with V. fetus.

	Animal number	During inoculation*		Post inoculation			Necropsy 5 days post inoculation**
		24 hrs.	48 hrs.	24 hrs.	48 hrs.	72 hrs.	
Test	1	+	+	-	-	-	-
	2	+	+	-	-	-	-
	3	+	+	-	-	-	-
	4	+	+	-	-	-	-
	5	+	+	-	-	-	-
	6	+	+	-	-	-	-
Total		6/6	6/6	0/6	0/6	0/6	0/6
Control	1	+	+	-	-	-	-
	2	+	+	-	-	-	-
Total		2/2	2/2	0/2	0/2	0/2	0/2

+ = growth of V. fetus; - = no growth of V. fetus in 5 days at 37 C.
* Cultures taken 24 and 48 hours after day 1 of the inoculation period
** No isolations of V. fetus were made from the uterus, cervix, blood, liver or spleen.

Experiment 9. Intravaginal inoculation of progesterone treated female guinea pigs. The pretrial vaginal cultures of the test and control animals were negative for V. fetus. The animals tolerated the inoculum well and remained alert and active. Some trauma was observed at the site of hormone injection, notably stiffness of the muscle and some tenderness. The vaginal cytology of the hormone treated test animals was characterized by a marked eosinophilia not observed in the vaginal smears of the control animals. The results of vaginal cultures taken during the study are given in Table 9. The vaginal cultures of the test and control animals were positive for V. fetus during the inoculation period. The organism was not isolated from the vagina of four of six test animals or from the vagina of one control animal 24 hours after the final inoculation was given. No isolations of V. fetus were made from any vaginal cultures taken at 48 and 72 hours post inoculation or from the vaginal cultures at necropsy.

The vaginal smears of the test animals taken in the post inoculation period showed a moderate to heavy cellular debris with numerous neutrophils and the eosinophilia noted at the start of the test period persisted. The vaginal flora comprised Gram positive and Gram negative cocci and bacilli. No organisms which morphologically resembled V. fetus were seen in Gram stained vaginal smears at any time. The cytology and flora of the vagina of the control animals were similar to those of the test group, however, there was no indication of eosinophilia.

No isolations of V. fetus were made from cultures of the uterus, cervix, blood, liver or spleen of the test or control animals when these organs were cultured at necropsy five days after the final intravaginal inoculation. The predominant microorganisms isolated from the vagina

Table 9. Vaginal cultures of progesterone treated guinea pigs during and after intravaginal inoculation with V. fetus.

	Animal number	During inoculation*		Post inoculation			Necropsy 5 days post inoculation**
		24 hrs.	48 hrs	24 hrs.	48 hrs.	72 hrs.	
Test	1	+	+	-	-	-	-
	2	+	+	-	-	-	-
	3	+	+	+	-	-	-
	4	+	+	-	-	-	-
	5	+	+	+	-	-	-
	6	+	+	-	-	-	-
Total		6/6	6/6	2/6	0/6	0/6	0/6
Control	7	+	+	+	-	-	-
	8	+	+	-	-	-	-
Total		2/2	2/2	1/2	0/2	0/2	0/2

+ = growth of V. fetus; - = no growth of V. fetus in 5 days at 37 C

* Cultures taken 24 and 48 hours after day 1 of the inoculation period

** No isolations of V. fetus were made from the uterus, cervix, blood, liver or spleen

of the test and control animals were E. coli, Pseudomonas aeruginosa, Proteus sp., Neisseria sp., Streptococcus sp. and diphtheroid organisms.

No abnormalities were detected in the histological studies of uterine tissue from any animal. The condition of the endometrium and uterine glands was compatible with the hormonal state of the uterus.

Experiment 10. Intrauterine inoculation with V. fetus during exogenous estrogen administration. All the test animals and sham operated controls tolerated the surgical procedure and anesthesia very well and showed no adverse after effects. The exogenous estrogen caused enlargement of the vulva and increased secretion of cervical-vaginal mucus. Hyperemia of the uterus was observed at laparotomy. The results of the cultures taken at necropsy from the animals euthanized at 24, 48 and 72 hours after inoculation are given in Table 10. Isolation of V. fetus was made from blood from the heart of three of four animals examined at each time period. The left and right uterine horns of all the animals examined 24 hours after inoculation were positive for V. fetus. Isolation of V. fetus was made from the saline washings of the uterine horns of these animals. Cultures taken from the left and right uterine horns of three of four animals examined 48 hours after inoculation were positive for V. fetus. The uterus of one animal (F) in this group was heavily contaminated with Proteus sp., and isolation of V. fetus was made only from the saline washings of the horns. The saline washings, from the left and right uterine horns of one animal (I), and from the left uterine horn of another (K), were negative for V. fetus 72 hours after inoculation.

Table 10. Cultures taken at necropsy following the intrauterine inoculation with V. fetus of ovariectomized estrogen treated guinea pigs.

Animal identification letters	Hours after inoculation: 24 A	24 B	24 C	24 D	48 E	48 F	48 G	48 H	72 I	72 J	72 K	72 L	Sham operated controls (2 animals)
Tissues cultured													
Blood (heart)	+	+	+	-	+	+	+	-	+	+	-	+	-
Uterus R. horn	+	+	+	+	+	-	+	+	+	+	+	+	-
L. horn	+	+	+	+	+	-	+	+	+	+	+	+	-
Saline wash R. horn	+	+	+	+	+	+	+	+	-	+	+	+	-
Cervix	+	+	+	+	+	+	+	+	+	+	+	+	-
Vagina	+	+	+	+	+	+	+	+	-	+	-	+	-
Liver	-	-	-	-	+	-	+	+	+	+	-	+	-
Bile	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	+	-	+	+	-	+	-	+	-
Peritoneum	-	-	-	-	+	-	+	+	-	+	-	+	-
Total positive cultures per animal	7	7	7	6	10	5	10	9	5	10	4	10	0

+ = V. fetus isolated
- = no growth of V. fetus after 5 days at 37 C
Tissues of animal F were contaminated with Proteus sp.

Similar cultures made on the remainder of the test animals in this group were positive for V. fetus.

Isolations of V. fetus were made from the cervical cultures of all the animals in every test group. The cultures from the vagina of animals in the 24- and 48-hour test groups were positive for V. fetus. In the 72-hour group V. fetus isolations were made from the vagina of two of the four animals.

No isolations of V. fetus were made from the liver, spleen and peritoneum 24 hours after inoculation. Isolations of V. fetus were made from these sites in three of four animals in the 48-hour test group. At 72 hours post inoculation V. fetus was isolated from the liver of three of four animals and from the spleen and peritoneum of two of four animals. No isolations of V. fetus were made from the bile of any animal examined.

The blood cultures of three of four animals in each time group were positive for V. fetus. Two of the positive blood cultures revealed non-motile organisms on dark-field examination which proved to be V. fetus when the blood cultures were subcultured on selective blood agar plates. The results of the viable cell counts of V. fetus performed on the washings from the right uterine horns of the animals are given in Table 11. This table also lists the secondary organisms isolated from the tissues of the animals at necropsy. A decrease was observed in the number of viable V. fetus cells in the right uterine horns of the animals examined at necropsy.

The animals tolerated the inoculum and remained active and alert throughout the entire test period. No anorexia was noted. No gross

Table 11. Cell counts of the right uterine horns and secondary organisms from the tissues of estrogen treated ovariectomized guinea pigs following intrauterine inoculation with *V. fetus*.

Animal	Viable count of *V. fetus* per ml (5.0 ml total)	Predominant secondary microorganisms and source of isolation
Total viable cells of *V. fetus* inoculated = 1.2×10^6		
	24 hours post inoculation	
A	4.9×10^2	*Proteus* sp.[1,4]; *Streptococcus* sp.[2]
B	6.1×10^2	*Streptococcus* sp.[5] ; *Staphylococcus* sp.[3]
C	8.0×10^2	*Streptococcus* sp.[3] ; *Staphylococcus aureus*[2]
D	9.8×10^3	*Streptococcus* sp.[3]
	48 hours post inoculation	
E	less than 100	*Neisseria* sp.[2,3]; *D. pneumoniae*[5]
F*	less than 100	*Proteus* sp.[1,2]; *Aerobacter* sp.[1]
G	less than 100	No predominant organisms
H	less than 100	No predominant organisms
	72 hours post inoculation	
I	less than 100	*Aerobacter* sp.[4] ; *Proteus* sp.[4]
J	less than 100	No predominant organisms
K	less than 100	*Streptococcus viridans*[2]
L	less than 100	No predominant organisms

Key to superscripts given to predominant organisms: 1 cervix; 2 left uterine horn; 3 right uterine horn; 4 vagina; 5 blood from the heart
*Proteus sp. isolated from the liver, spleen and peritoneal cavity

lesions were observed at necropsy and ovarian tissue was absent. The sham operated control animals were negative for V. fetus and showed no apparent gross changes in the reproductive system or other abdominal viscera.

Histological studies of the uterine horns from animals in the 24-hour test group showed neutrophilic infiltration of the uterine glands with some neutrophils in the lamina propria. Uterine tissues taken from animals in the 72-hour test group showed mild inflammation with some neutrophilic infiltration between the endometrial cells.

Experiment 11. Intrauterine inoculation with V. fetus during exogenous progesterone administration. The test and sham operated control animals tolerated the surgical procedure and anesthesia without any adverse aftereffects. The progesterone injections produced some muscle stiffness but this was not as pronounced as that described previously in the intact animals (Experiment 9). The vaginal cytology was typical of that produced by progesterone and the uterus of each animal was observed to be smaller and lacked the hyperemia present in the estrogen treated animals.

The results of cultures for V. fetus taken at necropsy 24, 48 and 72 hours after intrauterine inoculation are given in Table 12. Isolation of V. fetus was made from the blood of two of four animals 24 hours after inoculation and from all the animals in the 48- and 72-hour time intervals.

Isolations of V. fetus were made from the left and right uterine horns, the saline washings from these horns, the cervix, vagina, liver

Table 12. Cultures taken at necropsy following the intrauterine inoculation with V. fetus of ovariectomized progesterone treated guinea pigs.

Animal identification letters	Hours after inoculation 24 M	24 N	24 O	24 P	48 Q	48 R	48 S	48 T	72 U	72 V	72 W	72 X	Sham operated controls (2 animals)
Tissues cultured													
Blood (heart)	-	-	+	+	+	+	+	+	+	+	+	+	-
Uterus R. horn	+	+	+	+	+	+	+	+	+	+	+	+	-
L. horn	+	+	+	+	+	+	+	+	+	+	+	+	-
Saline wash													
R. horn	+	+	+	+	+	+	+	+	-	+	-	+	
L. horn	+	+	+	+	+	+	+	+	-	+	-	+	-
Cervix	+	+	+	+	+	+	+	+	+	+	+	+	-
Vagina	+	+	+	+	+	+	+	+	+	+	+	+	-
Liver	+	+	+	+	+	+	+	+	+	-	-	+	-
Bile	+	-	-	+	-	-	-	-	-	-	-	-	-
Spleen	+	+	+	+	+	+	+*	+	+	-	-	+	-
Peritoneum	-	-	-	-	-	-	-	-	-	+	+	+	-
Total positive cultures per animal	9	8	9	10	9	9	9	9	7	8	6	10	0

+ = V.fetus isolated
- = No growth of V.fetus in 5 days at 37 C.
* Splenic abscess found from which E. coli was isolated

and spleen of all animals examined 24 and 48 hours after inoculation. The spleen of one animal (S) contained an abscess from which E. coli was isolated. Twenty-four hours after inoculation V. fetus was isolated from the bile of two animals. Cultures made of the bile of the other two animals in the 24-hour group and from animals in the 48- and 72-hour groups did not result in the isolation of V. fetus.

Cultures of the left and right uterine horns, cervix, and vagina of all animals in the 72-hour test group were positive for V. fetus. The saline washings from the left and right uterine horns of two of four animals in the 72-hour group also were positive. Isolations of V. fetus were made from the liver and spleen of two of four animals in the 72-hour test group. The peritoneal cultures of animals examined at 24 and 48 hours post inoculation were negative for V. fetus, but V. fetus was isolated 72 hours post inoculation from the peritoneal cultures of three of four animals. No isolations of V. fetus were made from cultures taken from the sham operated control animals.

The results of viable cell counts of V. fetus made on the right uterine horn washings are given in Table 13. This table also lists the secondary microorganisms isolated from the reproductive systems and other organs cultured during the study. A reduction in the number of viable cells of V. fetus isolated from the right uterine horns was observed. Secondary organisms were confined to the vagina and distal aspect of the cervix of the animals examined. No secondary organisms were isolated from the uterine horns, blood, liver or spleen of any animal.

Table 13. Cell counts of the right uterine horns and secondary organisms from the tissues of progesterone treated ovariectomized guinea pigs following intrauterine inoculation with V. fetus.

Animal	Viable count of V. fetus per ml (5.0 ml total)	Predominant secondary microorganisms and source of isolation
Total viable cells of V. fetus inoculated = 6.6×10^6		
	24 hours post inoculation	
M	1.5×10^3	Coliform organisms[1,4]
N	2.6×10^2	E. coli[1,4]
O	3.0×10^2	E. coli[1,4]; Streptococcus sp.[1,4]
P	less than 100	Pseudomonas sp.[4]; E. coli[1,4]
	48 hours post inoculation	
Q	1.8×10^2	No predominant organisms
R	6.9×10^2	No predominant organisms
S*	less than 100	Coliform organisms[1,4]; Staphylococcus sp.[1,4]
T	6.2×10^2	Proteus sp.[1,4]; E. coli[4]
	72 hours post inoculation	
U	less than 100	No predominant organisms
V	less than 100	Proteus sp.[1,4]
W	No growth	No predominant organisms
X	less than 100	E. coli[1,4]; Pseudomonas aeruginosa[1,4]

Key to superscripts given to predominant organisms: 1 cervix; 2 left uterine horn; 3 right uterine horn; 4 vagina
*Splenic abscess; E. coli isolated

Apart from the splenic abscess previously described no other gross lesions were found at necropsy. Ovarian tissue was absent in all the animals examined.

Histological examination of the uterine tissues from all the test animals showed congestion of the lamina propria with slight lymphocytic infiltration. These changes were more pronounced in the right uterine horn.

Experiment 12. Intrauterine inoculation of pregnant guinea pigs. All the test animals and sham operated controls were vibrio-free at the start of the test. The surgical procedure and anesthesia were well tolerated and no adverse aftereffects were noted. The non-infected controls maintained normal pregnancy.

The test animals appeared listless and became inactive within 12 hours after inoculation. The hair coat became ruffled and harsh in appearance. Some anorexia was noted together with an increase in water consumption. There was no rise in body temperature. Six of the 11 test animals (1, 2, 3, 4, 9 and 11) aborted within 12 to 36 hours after inoculation and one (7) aborted between 36 and 48 hours post inoculation. Four animals (5, 6, 8 and 10) did not abort. Two of these animals (8 and 10) were allowed to go to term.

The abortions were preceded by the appearance of a bloody vaginal discharge which was initially reddish-brown in color. Later the discharge became bright red. Rapidly motile organisms were present in the discharge which when plated on selective blood agar plates proved to be V. fetus.

Two animals (1 and 2) which aborted were euthanized 24 hours

after inoculation. A single animal (7) was euthanized following abortion 48 hours after inoculation. The animals which survived abortion and were not euthanized (3, 4, 9 and 11) together with those allowed to term (8 and 10) developed a vaginal V. fetus infection which persisted for 72 hours. This infection was characterized by the isolation of decreasing numbers of V. fetus organisms toward the end of the 72-hour test period. No isolations of V. fetus were made from the vagina of these animals after four days. The vaginal cytology during this period comprised numerous neutrophils and erythrocytes with heavy cellular debris and some intact epithelial cells. Numerous vibrio-like organisms were seen in Gram stained vaginal smears taken 24 hours after inoculation. After 72 hours vibrio-like organisms were no longer seen in the vaginal smears although isolations of V. fetus were made from the vagina of test animals examined.

The results of cultures taken at necropsy on the euthanized dams are given in Table 14. Isolations of V. fetus were made from the uterus, cervix and vagina of each animal examined 24, 48 and 72 hours after inoculation. The bile and ovaries of each animal were negative for V. fetus. Culture of the spleen resulted in the isolation of V. fetus from one animal (1) in the 24-hour examination group and from another (6) in the 72-hour group. V. fetus was isolated from the blood of two animals (1 and 2) 24 hours after inoculation and from another (6) 72 hours after inoculation. The peritoneal cavity of one animal (1) was positive for V. fetus when cultured 24 hours after inoculation.

Gross lesions observed at necropsy of the dams were confined to

Table 14. Cultures taken at necropsy from gravid guinea pigs euthanized following intrauterine inoculation with V. fetus.

Date of inoculation: 6-2-70, 8.00 to 10.00 a.m.					
Time and date abortion complete	8.00 a.m. 6-3-70	11.00 a.m. 6-3-70	7.00 a.m. 6-4-70	No abortion	No abortion
Time and date of necropsy	11.00 a.m. 6-3-70	2.00 p.m. 6-3-70	10.00 a.m. 6-4-70	8.30 a.m. 6-5-70	10.00 a.m. 6-5-70
Animal number	1	2	7	5	6
Tissues cultured					
L. uterine horn	+	+	+	+	+
R. uterine horn	+	+	+	+	+
Cervix	+	+	+	+	+
Vagina	+	+	+	+	+
Liver	+	-	+	-	+
Bile	-	-	-	-	-
Spleen	+	-	-	-	+
Blood	+	+	-	-	+
Peritoneum	+	-	-	-	-
Ovary	-	-	-	-	-
Fetal tissues*	+	+	+	+	+
Total positive cultures per animal	9/11	6/11	6/11	5/11	8/11

+ = growth of V. fetus
- = no growth of V. fetus in 5 days at 37 C
* Further results of fetal cultures are given in Table 15

the uterus, cervix and vagina. In those animals which aborted before euthanasia the uterus was generally enlarged, flaccid and extremely hyperemic. The uterine cavity was filled with a mucoid blood-stained fluid. The cervix was relaxed and lacked tone. Tenacious blood-stained mucus occluded the cervical os. The vagina was filled with a similar blood-stained mucus. Gram stained preparations of this mucus revealed numerous neutrophils and erythrocytes with large masses of vibrio-like organisms clumped together (see Appendix C). The organisms were unevenly distributed throughout the smears and many high power fields were devoid of them.

The vaginal smears of the animals which aborted but were not euthanized showed some intact epithelial cells with moderate numbers of neutrophils and erythrocytes. Vibrio-like organisms were seen in only two vaginal smears.

The reproductive tracts of the two animals (5 and 6) which were euthanized without history of abortion differed in gross appearances. The right uterine horn of animal number 5 was enlarged, and hyperemic. The left uterine horn was enlarged hyperemic and congested about a small area of the endometrium. The uterine glands at this point appeared tortuous and the endometrial surface showed evidence of previous nidation, although no embryo was found. The uterus of animal number 6 contained three small embryos of approximately 0.5 cm crown rump length, one in the right uterine horn and two in the left uterine horn. The placental tissue was not well differentiated and was of a pulpy consistency. Petechial hemorrhages were seen on the uncut surface of the placenta. The uterine washings from both animals were grossly blood-stained and contained many

neutrophils. Occasional vibrio-like organisms were seen in Gram stained preparations of the uterine washings.

The results of the cultures taken from the aborted fetuses and the fetuses *in utero* are given in Table 15. The number of fetuses aborted by the dams ranged from one to six with a mode of three. The crown rump lengths of the fetuses ranged from 0.5 cm to 5.0 cm, the length of the fetuses from any one dam being somewhat uniform. The gross appearances of the fetuses varied from normal to macerated. Some aborted fetuses showed evidence of cannibalism by the dam.

Some of the fetuses had petechial hemorrhages of the skin surfaces. Other fetuses were reddish-brown in color with softer tissues than those of control guinea pig fetuses at the same state of gestation. Impression smears of the skin surfaces of the macerated fetuses showed numerous highly motile vibrio forms when examined by dark-field technique. These organisms were not found on impression smears of the skin surfaces of non-macerated fetuses. Some of the aborted fetuses showed signs of post-mortem lividity.

Isolation of *V. fetus* was made from the stomach contents of six aborted fetuses (from dams 6 and 7). Isolation of *V. fetus* was also made from the fetal thoracic and abdominal viscera and from the placentae of fetuses from seven of the dams. Isolation of *V. fetus* was made in every instance in which amnionic fluids, amnionic membranes or fetal blood were available for culture.

Accurate viable cell counts for *V. fetus* were not possible on the uterine fluids due to their mucoid nature and relatively small volumes. Direct culture of the fluids and infected uterine surfaces

Table 15. Bacterial flora of aborted fetuses and fetuses *in utero* following intrauterine inoculation of the dam with *V. fetus*.

	1	2	3	4	6	7	9	11
Number of fetuses aborted or *in utero*	3*	3*	6*	3*	3	3*	1*	1*
Average crown-rump length (cm)	3.3	4.5	4.5	5.0	0.5	5.0	2.5	3.0
Tissues cultured								
Stomach fluid	NA	+	NA	NA	EC	+	NA	NA
Viscera	+	+	+	+	EC	+	+	+
Placenta	+	+	+	NA	+	+	+	+
Amnionic fluid	NA	+	+	NA	EC	NA	NA	NA
Amnionic membrane	NA	+	+	NA	EC	NA	NA	NA
Blood	NA	+	NA	NA	EC	+	NA	NA

* Aborted
NA = not available for culture
+ = isolation of *V. fetus* from all fetuses cultured
EC = entire fetus cultured; *V. fetus* isolated

plated on selective blood agar resulted in a heavy, confluent and pure growth of V. fetus.

Histopathological examination of the tissues taken from euthanized dams examined following abortion showed superficial denudation of the endometrium with a severe inflammatory reaction characterized by polymorphonuclear and mononuclear leucocytic infiltration into the endometrium. Thrombi were observed in some of the uterine vessels and hemorrhage was present in the lamina propria. An acute patchy inflammatory reaction with edema was noted in the myometrium.

The placental tissues revealed extensive gross areas of necrosis and hemorrhage which varied in intensity. Fetal membranes were congested and necrotic with evidence of hemorrhage present.

Histological examination of euthanized animals which did not abort showed areas of inflammatory cell infiltration into the uterine glands and small hemorrhages into the lamina propria and some thrombosis. The placental tissues showed an acute inflammatory reaction with hemorrhage and areas of necrosis.

The two animals (8 and 10) in which gestation was to be maintained to term although pregnant at the time of inoculation showed no signs of a progressive pregnancy and were found non-pregnant when a necropsy was performed on the expected date of parturition. Cultures taken from the reproductive tract, liver, spleen, blood and the peritoneal cavity of these animals were negative for V. fetus.

The four animals which aborted but were not euthanized at the time of abortion (3, 4, 9 and 11) were subsequently euthanized 26 days after intrauterine inoculation. No isolations of V. fetus were made

from any of the tissues cultured. No gross lesions were found at necropsy in any of these animals, or the animals maintained to term. No changes were seen in tissues from the reproductive tracts of these animals when histopathological examination was made following necropsy.

DISCUSSION

The *in vitro* studies reported in the present work were attempted in an effort to determine the possible effect of erythritol, follicle stimulating hormone (FSH), estrone and progesterone on the growth of *V. fetus* in an artificial medium. These effects were assessed in the exponential phase of cell growth. A curve of change in optical density (OD) against time was substituted for the standard growth curve.

The standard bacterial growth curve described in textbooks of bacteriology (Burrows, 1959; Stanier, Douderoff and Adelberg, 1957) is based on the characteristic growth pattern derived by counting the number of viable cells in a growing culture over a period of time. The logarithm of the number of viable cells is then plotted against time. The use of OD to replace the need for repeated viable cell counts in studies of bacterial growth was found to be of value by Lyon, Hall and Costas-Martinez (1970). A standard of cell dry weight was used in the study of *Mycobacterium tuberculosis*. The growth curves derived in this study are recognized as not being absolute measures of bacterial cell numbers since neither the actual dry weight of the cells, the cell size nor the total cell count was determined. The reliability of the OD method of growth study was reported to improve with the use of synchronized cultures (Wilson and Miles, 1964). Synchronized cultures allow the subsequent increase in OD of the culture to be

proportional to the increase in cell numbers. This proportionality ceases to be a linear function when the OD reading exceeds 0.4 (Anon., 1957). The slopes of the linear plots obtained in Experiments 1 through 5 were calculated from the points derived during the interval when the growth of the cells was apparently logarithmic.

The OD of a culture is affected by changes in cell size. Most of these changes occur in the lag phase of growth and are accompanied by increased cellular metabolism. The actual lag refers to cell division rather than cell metabolism (Thimann, 1963). The size of the cell increases in the lag phase and usually reverts back as the actual exponential or log phase of growth begins.

Turbidometric methods were used in studies of possible growth factors for V. fetus by Trueblood and Tucker (1957) and Reich, Dunne, Bortree and Hokanson (1957). In all cases these authors compared the test growth curves with those of controls treated in an identical manner but without the growth factor or substrate added.

The choice of Brucella broth as a growth medium was made on the basis of its wide acceptance and value in the study of V. fetus (Reich, Morse and Wilson, 1956). A true minimal medium that will support the growth of all V. fetus isolants has not been defined although Alexander (1957) described a medium of brain liver heart, sodium chloride, phosphate buffer and 0.05% agar for the growth of ovine isolants of V. fetus. The erratic growth of V. fetus in a chemically defined medium was described by Fletcher and Plastridge (1963).

The metabolism of V. fetus was determined to be microaerophilic by May (1953). The organism was found to lack fermentative ability when

studies of possible energy sources were made by Alexander (1957). The inability of V. fetus to metabolize carbohydrates, especially dextrose, is noted in Bergey's Manual of Determinative Bacteriology (1957).

The possibility of erythritol as a growth factor for V. fetus was suggested by Herzberg (1966). The presence of erythritol in bovine fetal fluids was determined by Pearce et al. (1962). The position of erythritol in the metabolic pathway is not well understood. Hers (1958) proposed an intermediary function between D-erythrose and D-erythrulose in the pentose pathway. The possibility existed that if erythritol was a growth factor for V. fetus it could be injected into the guinea pig prior to inoculation and enhance the virulence of V. fetus. Keppie et al. (1965) used this technique to enhance the virulence of Brucella abortus in the guinea pig.

The results of Experiment 1 clearly indicated that, under the experimental conditions employed, erythritol was not a growth factor for V. fetus.

Osborne and Bourdeau (1955) reported the stimulatory effects of steroid hormones on the growth of V. fetus. Their study mentioned neither the method of addition of the steroid hormones to the culture medium nor an assay of the purity of the hormone preparations used. The extent of growth increase was measured by the depth of the subsurface layer of cells obtained in semi-solid thiol medium. Zemjanis and Hoyt (1960) reported an increase in the depth of the subsurface growth layer of V. fetus cultures to which a concentration of 0.005% 17, beta-estradiol was added. Concentrations of 0.1 to 0.2% 17,

beta-estradiol were inhibitory to V. fetus. In the present study no statistically significant change ($p = 0.05$) in the growth rate of V. fetus was obtained in media which contained concentrations of estrone or progesterone rangeing from 0.0005 to 0.01%. Under the experimental conditions employed neither estrone nor progesterone were growth factors for V. fetus. The analysis of the results of Experiment 4 indicated a statistically significant increase ($p = 0.05$) in the growth rate of V. fetus when combinations of estrone and progesterone were incorporated into the medium. The mechanism by which this increase in growth rate occurred was not determined. However, there was the possibility of a synergistic effect when the two hormones were combined. In view of the differences in methodology it was not possible to make a critical comparison of the results of Osborne and Bourdeau (1955) and Zemjanis and Hoyt (1960) with those obtained in this study.

One of the prime factors to be considered in such in vitro growth experiments as those reported in the present work was the extreme insolubility of the steroid hormones in an aqueous medium. Both estrone and progesterone are almost insoluble in water (Anon., 1952). This factor alone must influence the outcome of the experiments since no matter how great the quantity of hormone physically present in the medium the quantity that actually goes into solution remains extremely small and may not be increased by the mere addition of a greater quantity of hormone to the medium. The results shown in Tables 2, 3 and 4 are best considered from a qualitative rather than from a strictly quantitative point of view.

Earlier preliminary studies made by the author indicated that in

order to maintain the total quantities of the steroid hormones used in a completely soluble form, a level of organic solvent was required which was toxic to the test organism. For this reason a minimal volume of chloroform was used to add the required quantity of steroid hormone to the medium. The subsequent distillation of the solvent from the aqueous medium removed the possibility of toxicity to the test organism. As the chloroform was distilled off the bulk of the hormone then precipitated.

The precise level of soluble steroid hormones achieved in Experiments 2, 3 and 4 was not determined and the results must therefore be carefully interpreted. Any change in cell growth rate was due either to the amount of insoluble or soluble hormone present in the medium. There was also the possibility that both the soluble and insoluble factors could influence the growth of V. fetus. The nature of hormonal action in vivo is characterized by the extremely small quantities of hormone necessary to exert an effect on the target tissues (Harper, 1963). A similar situation may influence the growth of microorganisms in vitro when hormones are added to culture media. Studies of the effect of the hormones at a cellular level were not made; however, quantitative estimates of increased protein, DNA or RNA synthesis would be of value.

This study did not give any indication of actual breakdown of the hormones by microbial action or any indication of physical changes induced in the culture medium by the presence of the hormones. These factors would need to be determined by biological, physical and chemical assay. The in vitro aspects of this study were directed toward the

possibility that estrogen or progesterone either individually or in combination could influence the growth of V. fetus in vivo. It was interesting to note that the only significant growth response occurred in the estrone-progesterone combinations (Experiment 4) in which there was the possibility of a synergistic effect.

The relationship between the in vitro hormone levels achieved in this study and those which occurred naturally or were induced by the exogenous hormone administered in vivo can only be presumed (Nalbandov, 1964). Evidence of the direct effect of estrogen and progesterone upon cell components and growing tissues was given by Brooks, Leitheiser, DeLoecker and De Wever (1969); Hahn, Church, Gorbman and Wilmot (1968); and O'Malley, Aronow, Peacock and Dingman (1968). Studies made by these authors indicated significant increases in protein synthesis in uterine microsomal supernatants in vitro (Brooks et al., 1969) induced by estrone sulphate and progesterone; and estrogen induced increase of t-RNA formation and the production of new types of RNA in the chick oviduct (Hahn et al., 1968; O'Malley et al., 1968).

The statistical analysis of the results of Experiment 5 indicated that the addition of FSH to the culture medium in concentrations ranging from 0.0005 to 0.01% did not affect the growth rate of V. fetus.

Although certain combinations of estrone and progesterone significantly increased the growth rate of V. fetus in vitro, the possibility that estrogen and progesterone, either singly or in combination, had the same effect in the guinea pig was not evident in the present study. It is known that estrogen can induce an antibacterial state in the uterus and progesterone produces changes in which the uterus is

more susceptible to bacterial infection (Hawk, 1958). Whether these hormonal influences apply to V. fetus infection requires further research. Black et al. (1953), in studies of the response of the rabbit uterus to V. fetus infection, failed to demonstrate any of the changes induced by pyogenic organisms. Although V. fetus was isolated from the inoculated uterine horns of estrous and pseudopregnant rabbit uteri the pathological response of the tissues was minimal.

The in vivo studies reported here made use of the vaginal route of inoculation although Ristic and Morse (1953) reported this route to be the least effective. Justification for the use of the intravaginal route of inoculation was based on the normal venereal route of infection in cattle. An attempt was made to duplicate this venereal route of infection as closely as possible in the guinea pig. Table 6 shows a relative lack of persistence of V. fetus in the vagina of the guinea pig even when the organism was introduced in large numbers. Vaginal cultures taken 72 hours after inoculation were negative for V. fetus in four of six animals regardless of the hormonal condition of the reproductive tract. Five days post-inoculation all the vaginal cultures were negative. This was in direct contrast with the infection in the heifer in which V. fetus was reported to persist in the vagina for as long as eight months (VanDeplassche et al., 1956).

The bacteriological and histological findings made after intravaginal inoculation of the intact guinea pig (Experiment 6) gave no indication that V. fetus entered the uterus. The possibility of uterine invasion of shorter duration, less than five days, was not ruled out. An experiment in which larger numbers of animals are used and examined

bacteriologically at closer intervals would be of value. The absence of a contaminating microflora in the uterus at necropsy contrasted with a vaginal flora of Pseudomonas sp. and E. coli apparently indicated an effective uterine defense mechanism in which the cervix acted as a barrier to direct infection of the uterus.

The use of exogenous estrogen in an attempt to increase the susceptibility of the guinea pig to V. fetus was reported by Adler (1953) and by Power (1954). Experiment 8 was an attempt to repeat the work of Adler (1953). The results of bacteriological studies of the estrus induced guinea pigs reported in Table 8 were very similar to the results of Experiment 6 in which guinea pigs with normal cycles were used. A lack of persistence of the organism in the vagina was again observed with some indication of an even more rapid rate of clearance. These results were in direct contrast with those of Adler (1953) who reported a 50% efficiency in the rates of isolation of V. fetus from guinea pigs inoculated intravaginally with infected bull semen and from animals inoculated with pure V. fetus cultures. Adler (1953) relied on phase contrast microscopy to establish the identity of V. fetus from the uterine tissues of guinea pigs. The presence of motile organisms, other than V. fetus, in the cultured tissues was a possibility not discussed by the author. Power (1954) also reported data in direct contrast with that of the present study and successfully infected three of six guinea pigs with infected bull semen inoculated into the vagina. Two of the negative animals were found to be pregnant. The results of Experiment 8 were supported by the data of Robinson et al. (1956) who reported failure in attempts to infect two groups of estrus induced guinea pigs with intravaginal inoculations of V. fetus.

The apparent dichotomy between the results of Adler (1953) and Power (1954) and those reported by Robinson et al. (1956) together with the data reported in this study shows an obvious need for further work in the study of V. fetus infection in the non-gravid female guinea pig. These studies should include an increase in the numbers of animals used, accurate identification of the V. fetus isolants and due regard for the possible loss of virulence in laboratory maintained cultures of V. fetus.

Exogenous progesterone apparently had little effect to increase the susceptibility of the female guinea pig to intravaginal inoculation with V. fetus. The organism failed to persist in the vagina (Table 9) although V. fetus was isolated from three animals 24 hours after the final intravaginal inoculation was given. Histological evidence of uterine invasion was absent and cultures taken from the uterus at necropsy were negative for V. fetus.

It is of interest to note that Manclark and Pickett (1965a) were unable to detect the formation of either mucosal or humoral antibody following the intravaginal inoculation of the virgin female guinea pig with live cells of V. fetus. The pattern of the immune response of the guinea pig to V. fetus infection was influenced by the route of inoculation, the type of antigen used and the hormonal condition of the reproductive tract. The highest antibody titers reported by Manclark and Pickett (1965a) were obtained when live V. fetus cells were inoculated intraperitoneally into pseudopregnant guinea pigs. In this case both humoral and mucosal antibodies were detected. An explanation for the absence of mucosal or humoral antibody in the virgin

guinea pig, following intravaginal inoculation with live V. fetus cells, may be a lack of antigen persistence as evidenced by the results of Experiments 6, 8 and 9.

The use of ovariectomized guinea pigs in the study of the intrauterine route of inoculation was made to permit the appraisal of the effect of estrogen and progesterone in vivo. The contribution of estrogen from the adrenals to compensate for the estrogen normally produced by the ovary was not considered significant in a system in which exogenous estrogen was administered to ovariectomized guinea pigs. Although the quantitative levels of estrogen induced by the hormone injections were not determined, the gross appearance of the reproductive tract, the vaginal cytology and the histological appearance of the uterus were compatible with estrogen stimulation. Previous studies of V. fetus infection in which an intrauterine route of inoculation was used include the works of Black et al. (1953) in the estrous and pseudopregnant rabbit and the work of Osburn et al. (1969) in the cow. The present work is the first reported use of the intrauterine route of inoculation of V. fetus in the guinea pig and the first study to define the V. fetus isolant used as V. fetus var. venerealis.

The results of the bacteriological examinations of the non-pregnant animals (Tables 8 and 10) inoculated by the intrauterine route were in contrast to those previously described in Experiments 6, 8 and 9 in which the intravaginal route of inoculation was used. After introduction into the lumen of the uterus V. fetus spread throughout the reproductive system within the first 24 hours post inoculation. The organism was also isolated

from the blood of the animals. Within 48 hours the liver and spleen were involved and the organism still persisted in the reproductive tract. Cultures taken 72 hours after inoculation showed an apparent clearance of the organism from the reproductive system and the spleen. Isolations of V. fetus from the liver and spleen of non-gravid guinea pigs inoculated intraperitoneally were reported by Smith (1923). The isolation of V. fetus var. venerealis from the blood of a non-gravid guinea pig has not been described previously and constitutes an important finding in the present work since V. fetus var. venerealis was presumed to localize in the reproductive tract. The possibility of bacteremia as a means for the systemic spread of V. fetus in the guinea pig was suggested by Smith (1919) and Ristic et al. (1954a) but was apparently not followed up by these workers in their later studies of V. fetus infection in the guinea pig. V. fetus var. venerealis infection in the cow was considered to be a localized infection centered in the reproductive tract (Manclark and Pickett, 1965a). No data are presently available on studies designed to determine if the organism ever enters the circulatory system of the cow.

The cultural data obtained from the progesterone treated ovariectomized guinea pigs (Table 12) after intrauterine inoculation were similar to that described from the estrogen treated animals. A notable difference was the isolation of V. fetus from the gallbladder of two animals examined 24 hours after intrauterine inoculation. Previous isolation of V. fetus from the gallbladder of the guinea pig was reported by Ristic et al. (1954). This followed the inoculation of a mixed culture of ovine and bovine isolants of V. fetus. The ovine isolant

designated strain K was possibly of the intestinalis variety. The isolation of V. fetus var. intestinalis from the gallbladder of the ewe is not uncommon and formed part of a study by Firehammer, Lovelace and Hawkins (1962).

The viable cell counts of V. fetus obtained from the saline washings of the right uterine horns of the estrogen and progesterone treated animals gave no indication that the organisms reproduced in the uterus. Failure to ligate the left uterine horn prior to inoculation allowed the organisms to gain direct access to the left uterine horn via the cervix. This precluded the use of the left horn of the uterus as a control tissue. An increase in V. fetus cell numbers could only be proven by count of the total number of cells throughout the body of the host. This was not attempted. In Experiments 10 and 11 the reduction in the viable cell count from the right uterine horn was possibly due to the spread of the organisms throughout the body. The organisms appeared to spread more rapidly in the estrogen treated animals than in the progestrone treated animals. It is speculative at this time to consider this as an effect of hormonal mediation. The possibility of a uterine clearance mechanism under the control of the endocrine system cannot be ruled out; however, the present study lends support to the opinion of Black et al. (1953) that V. fetus is not affected by such a mechanism.

The failure of V. fetus to persist in the non-gravid guinea pig uterus and vagina under the experimental conditions employed in this series of tests was probably due to an absence of host specificity and lends support to the earlier findings of Smith et al. (1920) in

which V. fetus infection in the guinea pig was described as transitory. The absence of any naturally occurring deaths and the lack of significant uterine lesions among the test populations (Experiments 6, 8, 9, 10 and 11) further supported the opinion of Smith et al. (1920) that V. fetus was not pathogenic for the non-gravid guinea pig. This opinion was reiterated by Manclark and Pickett (1965a).

The isolation of bacterial species, other than V. fetus, from the uterus of the test animals examined after intrauterine inoculation (Experiments 10 and 11) contrasted with the negative uterine cultures obtained when the intravaginal route of inoculation was used (Experiments 6, 8 and 9). The presence of these secondary isolants (Tables 11 and 13) could be due to the trauma of surgery and the possible post surgical debilitation of the animals; however, no secondary bacterial isolations were made from the non-infected sham operated control animals.

The experimental evidence (Experiments 10 and 11) indicated the passage of V. fetus from the uterus to the cervix and then into the vagina. In previous Experiments (6, 8 and 9) and in the report of Ristic and Morse (1953) V. fetus was unable to survive in the vagina or to pass through the cervix when inoculated intravaginally into the non-gravid guinea pig. It is possible that when placed directly into the bacteria-free non-gravid uterus V. fetus is able to survive and pass from the uterus through the cervix into the vagina. The ability of the organism to survive may be due to the absence of microbial competition for available substrates in the bacteria-free uterus. The differences in the chemicophysical environment of the uterus and that of the vagina may also be determining factors in the survival of V. fetus in the

reproductive tract. The continued passage of V. fetus from the uterus provides a continual reinfection of the vagina which persists until the uterine infection is cleared. The open cervix must provide the portal of entry by which the vaginal flora gain access to the uterine horns. The present study did not include the intrauterine inoculation of intact non-pregnant guinea pigs.

The investigations of Smith (1918; 1919 and 1923) did not include studies with pregnant guinea pigs. The absence of pathological change and the failure to isolate V. fetus four days after intraperitoneal inoculation led Smith (1918) to conclude that the guinea pig was refractory to V. fetus infection. Ristic and Morse (1953) reported abortions in guinea pigs one to 12 days after oral, intraperitoneal and intravaginal inoculation but, as reported earlier, these workers used a mixed inoculum of bovine and ovine isolants. In the present study abortion occurred in seven of 11 animals within 36 hours after intrauterine inoculation with V. fetus var. venerealis. This interval is shorter than that observed by Robinson et al. (1956) in which pregnant guinea pigs aborted four days after intraperitoneal inoculation with infected bovine semen. Osburn et al. (1969) noted abortion five days following the intrauterine inoculation of V. fetus in the cow during the second trimester of pregnancy. Cows in the third trimester of pregnancy aborted between nine and 20 days after similar inoculation. Whether this apparent resistance of the bovine fetus to V. fetus infection applies equally to the guinea pig is not known.

It is possible that V. fetus must first gain access to the uterus in order for abortion to occur in which case the route of infection is

an important factor. The isolation of the organisms from the blood of the animals which aborted (Experiment 12) and from the other non-pregnant animals previously described emphasizes the importance of the circulatory system in the spread of the infection from the uterus. The role of the circulatory system in the establishment of the naturally acquired infection in the cow, in which the organisms are inoculated into the vagina or into the cervix at artificial insemination, appears minimal.

The gross appearance of the aborted fetuses (Experiment 12) closely resembled that described by Ristic and Morse (1953). The bacterial examination of the aborted fetuses (Experiment 12) indicated that V. fetus had gained access to the stomach, viscera and blood regardless of the normal appearance of some specimens (see Appendix C). The macerated appearance of some of the fetuses was apparently related either to the extent of bacterial invasion or was due to the actual uterine position of the fetus in relation to the inoculum. Care was taken to avoid the direct inoculation of any fetus in utero; however, there was an initial concentration of the inoculum around the injection site and this may have caused the death of the fetus nearest to the site of injection. The mortality due to V. fetus infection among chicken embryos was reported between 10.7% and 85.7% by White, Ristic and Sanders (1958). The response was greatest when a human isolant of V. fetus was tested. There was no correlation between embryonic mortality and the number of viable cells in the inoculum.

The histopathologic changes observed in Experiment 12 were comparable with those described in the pregnant guinea pig by Ristic

et al. (1954a) and to the histopathologic changes observed in the heifer following V. fetus infection (Dozsa, Olson and Campbell, 1960). The presence of edema, inflammatory cell reaction and vessel thrombosis in the uterus of the cow as a result of V. fetus infection was described by Simon and McNutt (1957).

The work reported here does not eliminate the possibility of endotoxic action as a cause for the observed abortions since whole cells of V. fetus in the growth medium were used as the inoculum. Osborne (1965) reported the incidence of abortion induced by V. fetus endotoxin in the goat, cow, ewe and rabbit. The histopathology of the uterus of the above animals was similar to that found in naturally occurring infections and in the pregnant animals described in Experiment 12. The gross findings noted by Osborne (1965) in the heart, lungs, and liver of calves following the injection of V. fetus endotoxin were not apparent in the guinea pigs which were examined after abortion (Experiment 12). The possibility of differences in the responses of various animal species to endotoxins must be considered. The role of endotoxin as the cause of the infertility and abortion observed as the result of V. fetus infection was discounted by Dennis (1967). This author determined the toxicity of the endotoxins of pathogenic isolants of V. fetus and saprophytic vibrios and found them to be similar in potency. Dennis (1967) suggested a need to investigate causes other than endotoxic activity to account for the pathogenicity of V. fetus.

In Experiment 7 the animals were mated after intravaginal inoculation during the proestrus period. There was a significant difference ($p = 0.05$) in the number of offspring which survived for longer

than 30 hours in the control population when compared with the number of survivors in the test population. (Table 7 and Appendix A.) Significantly larger litters ($p = 0.05$) were produced by females in the control population than those produced by the test population (Table 7 and Appendix A). The number of stillborn offspring was higher in the control population than in the test population. This was probably a result of increased litter size. The stillborn offspring of both populations frequently weighed more than 75 grams, a birthweight which increased the trauma to the young at birth.

The results shown in Table 7 did not indicate "infertility" in the test or control groups; however, there was an indication that the presence of V. fetus in the vagina at the time of mating could subsequently affect the embryos either before or after nidation. At what stage in the gestation period V. fetus affects the embryo or fetus is not known. Ulberg and Burfening (1967) reported the effects of an adverse uterine environment on the embryo. They showed a direct relationship in the type and duration of stress to the ultimate survival of the embryo or the fetus. The possibility that V. fetus produced an adverse uterine environment in the guinea pig is worthy of consideration. The change induced in the intrauterine environment could be slight, in which case the fetus developed to term but died shortly after birth. A more severe change in the intrauterine environment could result in early abortion, resorption of the embryo or apparent failure of nidation. The possibility of a severe change was reinforced by the apparent complete resorption of the embryos of the two animals selected to go to term in Experiment 12. The predilection of V. fetus

for the gravid uterus was clearly shown in Experiment 12 and the results of this experiment support the findings of Manclark and Pickett (1965a) in this regard.

This work has attempted to describe the effects of V. fetus var. venerealis in the gravid and non-gravid guinea pig. The transitory nature of the infection and the absence of lesions in the non-gravid guinea pig, whether treated with steroid hormones or not, rendered the study of V. fetus infection in such a model difficult and produced inconclusive results. There is an indication that the steroid hormones (estrone and progesterone) may act as growth factors in vitro but no definite conclusion can be made that this indication holds true in vivo. The value of the non-gravid guinea pig as a diagnostic tool for the presence of V. fetus infection in cattle was not substantiated by this study. A diagnostic procedure which makes use of the intraperitoneal inoculation if the pregnant guinea pig seems feasible but more work is needed in this area.

The abortions observed in this study and those noted by the other workers cited in the text do not explain the "infertility" manifested by V. fetus infection in the cow. The refractory response of the guinea pig to infection by V. fetus is considered to be due to the strict host specificity of V. fetus var. venerealis which allows the fullest expression of its virulence solely in the reproductive system of the cow and renders an accurate study of such factors as pathogenicity, infectivity and antigenicity difficult, if not impossible in other animal species.

SUMMARY

This study was undertaken to determine the influences of specific endocrine factors on the growth of V. fetus var. venerealis in vitro and to relate any effects to similar factors in non-gravid and gravid guinea pig exposed to V. fetus var. venerealis infection. The study also included an appraisal of routes of inoculation and the general features of V. fetus var. venerealis infection in the female guinea pig.

1. The addition of erythritol, follicule stimulating hormone, estrone or progesterone to culture medium failed to give statistically significant evidence of growth stimulation of V. fetus var. venerealis in vitro.

2. The addition of estrone in combination with progesterone to culture medium provided statistically significant evidence ($p = 0.05$) of an increase in the growth rate of V. fetus var. venerealis in vitro.

3. Non-gravid guinea pigs with normal cycles and non-gravid guinea pigs treated with exogenous estrogen or progesterone failed to develop a prolonged vaginal or a systemic infection when inoculated intravaginally with V. fetus var. venerealis. No histopathologic lesions were seen in the reproductive tract.

4. Proestrus females inoculated with V. fetus var. venerealis intravaginally and then mated showed significant decreases ($p = 0.05$) in litter size and in numbers of offspring surviving 30 hours postpartum.

5. Ovariectomized guinea pigs treated with either exogenous estrogen or progesterone and inoculated with V. fetus var. venerealis by the intrauterine route developed a transitory but apparently not progressive infection of the uterus, cervix and vagina. The infection became systemic and involved the blood, liver, spleen, gallbladder and peritoneum. A hormonal influence on the course of the infection was not established. No histopathologic changes were seen in the reproductive tract.

6. Gravid guinea pigs inoculated by the intrauterine route with V. fetus var. venerealis aborted within 24 to 30 hours post inoculation. The aborted fetuses were infected and V. fetus var. venerealis was isolated from the stomach fluid, viscera, blood and body surfaces. Isolation of V. fetus var. venerealis was made from the fetal maternal membranes and from the uterus, cervix, vagina, liver, spleen, blood and peritoneum of the dams. The animals which did not abort showed signs of apparent fetal resorption and only transitory infection of the vagina following intrauterine inoculation with V. fetus var. venerealis. Histopathological examination of the uterus and placenta of the guinea pigs which aborted showed evidence of necrosis, thrombosis, hemorrhage, edema and inflammatory reaction.

APPENDIX A

Statistical Analyses; One Way Analysis of Variance (Steel and Torrie 1960).

Experiment 1. Erythritol as a growth factor.

Concentration per ml					
0.06	1.0	3.0	5.0	10.0	Controls
137	172	193	167	188	200
140	167	172	184	174	172
277	339	365	351	362	372

Total SS = 3928

Treatment SS = 3054

Error SS = 874

Analysis of variance

	df	SS	MS	F
Between groups	5	3054	610.8	4.11
Within groups	6	874	145.6	

F_{tab} at p = 0.05 is 4.39; at p = 0.01 is 8.75.

Reject H_o of erythritol effect.

Experiment 2. Estrone as a growth factor.

Concentration percent							
0.01	0.009	0.007	0.005	0.003	0.001	0.0005	Controls
595	580	442	500	525	535	590	500
590	580	538	536	538	514	535	450
1185	1160	980	1036	1063	1049	1125	950

Total SS = 32575
Treatment SS = 24237
Error SS = 8338

Analysis of variance

	df	SS	MS	F
Between groups	7	24237	3422	3.32
Within groups	8	8338	1042	

F_{tab} at p = 0.05 is 5.19; at p = 0.01 is 11.39.
Reject H_o of estrogen effect.

Experiment 3. Progesterone as a growth factor.

Concentration percent

0.01	0.009	0.007	0.005	0.003	0.001	0.0005	Controls
445	524	500	375	400	433	380	413
445	448	445	366	380	400	343	415
890	972	945	741	780	833	723	828

Total SS = 34984
Treatment SS = 24110
Error SS = 10874

Analysis of variance

	df	SS	MS	F
Between groups	7	24100	3444	2.53
Within groups	8	10874	1359	

F_{tab} at p = 0.05 is 3.50; at p = 0.01 is 6.37

Reject H_o of progesterone effect.

Experiment 4. Estrone and progesterone as combined growth factors.

Concentration estrone/progesterone (%)

0.001/0.009	0.003/0.007	0.005/0.005	0.007/0.003	0.009/0.001	Control
539	650	769	684	758	572
500	600	700	746	693	518
615	600	700	676	790	500
1654	1850	2169	2106	2241	1590

Total SS = 149926
Treatment SS = 117717
Error SS = 32209

Analysis of variance

	df	SS	MS	F
Between groups	5	117717	23543	8.76
Within groups	12	32209	2684	

F_{tab} at p = 0.05 is 3.11; at p = 0.01 is 5.06. Accept H_o of estrone/progesterone effect.

$S_{\bar{x}}$ = 32.74 Duncan's multiple range test (p = 0.05)

	2	3	4	5	6
SSR	3.08	3.23	3.33	3.36	3.40
LSR	100.80	105.70	109.00	110.00	111.30

Concentration estrone/progesterone (%)

Control	0.001/0.009	0.003/0.007	0.007/0.003	0.005/0.005	0.009/0.001
530	551	616	702	723	747

Experiment 5. FSH as a growth factor

Concentration (%)					
0.01	0.005	0.003	0.001	0.0005	Controls
10	9	8	9	9	10
9	9	9	9	9	9
8	6	9	9	9	9
27	24	26	27	27	28

Total SS = 9
Treatment SS = 4
Error SS = 5

Analysis of Variance

	df	SS	MS	F
Between groups	5	4	0.80	1.95
Within groups	12	5	0.41	

F_{tab} at p = 0.01 is 5.06; at p = 0.05 is 3.11

Reject H_o of FSH effect

Slopes derived from V. fetus growth curves in Experiments 1 to 5

Experiment 1. Erythritol as a growth factor

	Concentration (mgm/ml)					
	0.06	1.0	3.0	5.0	10.0	Control
A	1.37	1.72	1.93	1.67	1.88	2.00
B	1.40	1.67	1.72	1.84	1.74	1.72
$\bar{x}$ =	1.385	1.695	1.825	1.755	1.810	1.860

Experiment 2. Estrone as a growth factor

	Concentration (%)							
	0.01	0.009	0.007	0.005	0.003	0.001	0.0005	Control
A	0.595	0.580	0.442	0.500	0.525	0.535	0.590	0.500
B	0.590	0.580	0.538	0.536	0.538	0.514	0.535	0.450
$\bar{x}$ =	0.593	0.580	0.490	0.518	0.531	0.524	0.512	0.475

Experiment 3. Progesterone as a growth factor

	Concentration (%)							
	0.01	0.009	0.007	0.005	0.003	0.001	0.0005	Control
A	0.445	0.524	0.500	0.375	0.400	0.433	0.380	0.413
B	0.445	0.448	0.445	0.366	0.380	0.400	0.343	0.415
$\bar{x}$ =	0.445	0.486	0.472	0.370	0.390	0.416	0.361	0.414

Experiment 4. Estrone and progesterone as combined growth factors

	Concentration (%)					
	0.001/0.009	0.003/0.007	0.005/0.005	0.007/0.003	0.009/0.001	Control
A =	0.539	0.650	0.769	0.684	0.758	0.572
B =	0.500	0.600	0.700	0.746	0.693	0.518
C =	0.615	0.600	0.700	0.676	0.790	0.500
$\bar{x}$ =	0.551	0.616	0.723	0.702	0.747	0.530

Experiment 5. FSH as a growth factor

	Concentration (%)					
	0.01	0.005	0.003	0.001	0.0005	Control
A =	1.00	0.90	0.80	0.90	0.90	1.00
B =	0.90	0.90	0.90	0.90	0.90	0.90
C =	0.80	0.60	0.90	0.90	0.90	0.90
$\bar{x}$	0.90	0.80	0.86	0.90	0.90	0.94

Experiments 2, 3 and 4. Effects of chloroform treated medium on the growth of V.fetus var. venerealis.

chloroform treated medium	untreated medium
545	555
524	525
1069	1080
$\bar{x}$ = 534.5	μ = 540.0

$$t_{calc} = \frac{\bar{x} - \mu}{s_{\bar{x}}}$$

$$t_{calc} = \frac{534.5 - 540.0}{10.47} = \text{less than } 1.00$$

$t_{0.05}$ = 6.314 is greater than 1.00 therefore reject H_o of chloroform effect.

Experiment 7. Intravaginal inoculation of the normal estrous guinea pig with subsequent mating.

Reproduction efficiency of females

a. Litter size

Test group: $\bar{x} = 2.30$ Control group: $\bar{x} = 3.30$

$(\bar{x} - x_1)^2 = 26.10$ $(\bar{x} - x_2)^2 = 26.10$

Pooled estimate of variance

$$s^2 = \frac{26.10 + 26.10}{10 + 10 - 2} = 2.9$$

$SD = 0.538$

$t_{calc} = 4.12$

$t_{0.05} = 1.73$ df = 18

Therefore reject H_0 of no difference

b. Young surviving more than 30 hours post-partum

Test group: $\bar{x} = 1.30$ Control group: $\bar{x} = 2.80$

$(\bar{x} - x_1)^2 = 26.00$ $(\bar{x} - x_2)^2 = 23.60$

Pooled estimate of variance

$$s^2 = \frac{26.00 + 23.60}{10 + 10 - 2} = 2.75$$

$SD = 1.66$

$t_{calc} = 2.05$

$t_{0.05} = 1.73$ df = 18

Therefore reject H_0 of no difference

APPENDIX B

Some notes on the female guinea pig (Cavia porcellus L.).

Age of puberty:	45 to 70 days
Minimum breeding age:	12 weeks
Minimum breeding weight:	450 grams
Length of estrus cycle:	16 days $\pm$ 2 days
Duration of estrus:	6 to 15 hours
Site of semen deposition:	Uterus
Ovum cleavage to blastocele:	5 to 6 days
Nidation occurs:	6 days
Gestation period:	58 to 75 days. (Average 64 to 68 days)

Both ovaries are active in each cycle

Source: Kaplan, H. M. Physiological Data for Common Laboratory Animals. Teklad Inc. Monmouth, Illinois.

APPENDIX C

Fig. 1. Experiment 7. Uterus, fetuses and placentas from animal number 2. The uterine wall is thin and various stages of fetal decomposition are evident.

Fig. 2. Experiment 7. Enlargement of fetus from animal number 2. The tissues were firm with the exception of those of the extremities.

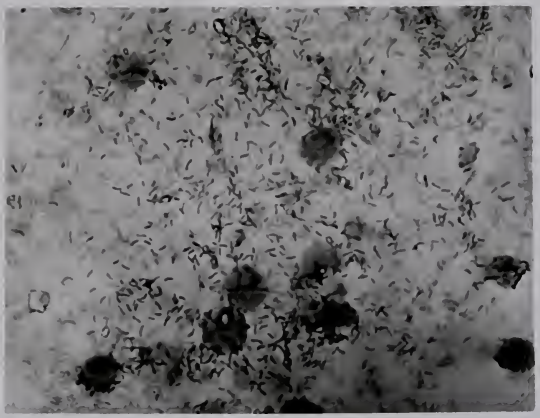

Fig. 3. Experiment 10. Vibrio fetus in a 24 hour broth culture of blood from the heart of an infected mature female guinea pig. Gram stain .X 1250.

Fig. 4. Experiment 11. Vibrio fetus in a 24 hour broth culture of bile from an infected mature female guinea pig. Gram stain .X 1250.

Fig. 5. Experiment 12. Vaginal smear from a guinea pig which aborted. Large numbers of Vibrio fetus cells are present in the neutrophil in the center of the field. Gram stain. X 1250.

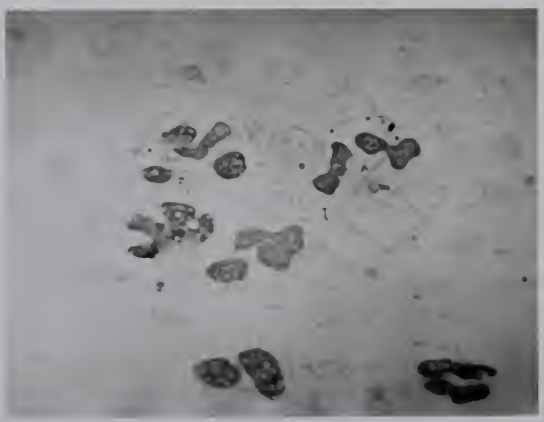

Fig. 6. Experiment 12. Another area of the same vaginal smear (above). Few Vibrio fetus cells are seen. Gram stain. X 1250.

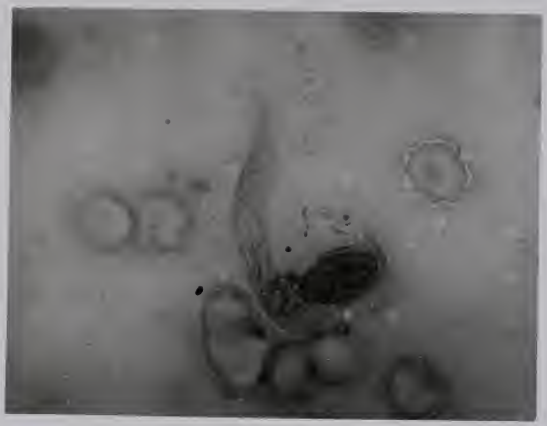

Fig. 7. Experiment 12. Vibrio fetus (center) present in an impression smear of the stomach of an aborted fetus. Gram stain. X 1250.

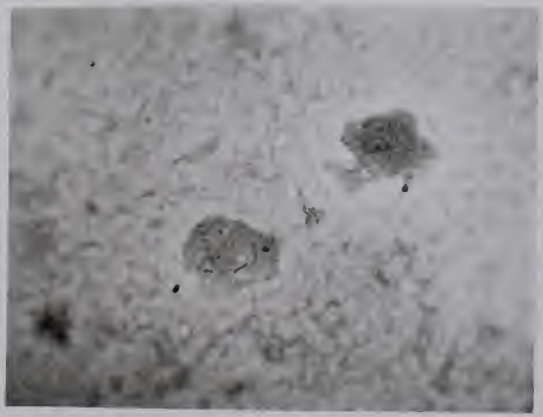

Fig. 8. Experiment 12. Vibrio fetus (center) present in an impression smear of the amnion of an aborted fetus. Gram stain. X 1250.

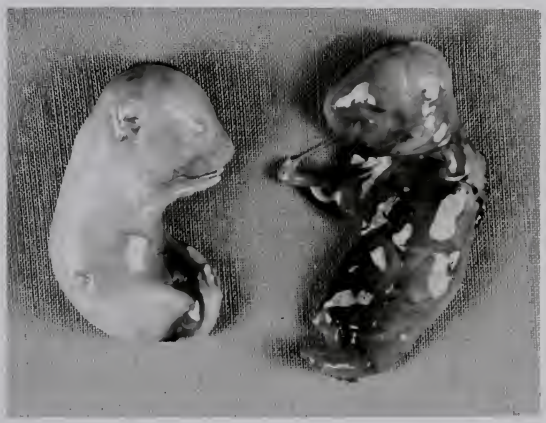

Fig. 9. Experiment 12. Fetuses aborted by the same female 24 hours after intrauterine inoculation with Vibrio fetus.

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BIOGRAPHICAL DATA

Anthony F. Walsh was born December 17, 1930, at London, England. In June 1946 he was graduated from St. Ignatius' College. He followed a career as a medical technologist in England before immigrating to the United States in 1956.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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